HER2 Status in Colorectal Cancer: Its Clinical Significance and the Relationship between HER2 Gene Amplification and Expression

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Abstract
This study aimed at determining the incidence and clinical implications of HER2 status in primary colorectal cancer (CRC). HER2 status was investigated in two retrospective cohorts of 365 consecutive CRC patients (cohort 1) and 174 advanced CRC patients with synchronous or metachronous distant metastasis (cohort 2). HER2 status was determined by performing dual-color silver in-situ hybridization (SISH), mRNA in-situ hybridization (ISH), and immunohistochemistry (IHC). The incidence of HER2 protein overexpression (IHC 2+/3+) was approximately 6% (22 of 365 in cohort 1; 10 of 174 in cohort 2). HER2 gene amplification was observed in 5.8% of the patients from cohort 1 and 6.3% of the patients from cohort 2. HER2 gene amplification was more frequently observed in CRCs located in the rectum than in the right and left colon (P = 0.013 in cohort 1; P = 0.009 in cohort 2). HER2 status, determined by IHC, ISH, and dual-color SISH, was not significantly associated with aggressive CRC behaviour or patients’ prognosis in both the cohorts. Of the combined cohort with a total of 539 cases, the concordance rate was 95.5% between dual-color SISH and IHC detection methods. On excluding equivocally immunostained cases (IHC 2+), the concordance rate was 97.7%. HER2 mRNA overtranscription, detected by ISH, significantly correlated with protein overexpression and gene amplification (P<0.001). HER2 gene amplification was identified in a minority of CRC patients with high concordance rates between dual-color SISH and IHC detection methods. Although HER2 status did not predict patients’ prognosis, our findings may serve as a basis for future studies on patient selection for HER2 targeted therapy.

Introduction
Despite advances in surgical techniques and adjuvant chemotherapeutic regimens, colorectal cancer (CRC) remains one of the major leading causes of cancer-related deaths world over [1,2]. Further improvements in understanding tumor biology and identifying oncogenic drivers have led to the development of new therapeutic targets [2,3]. Therefore, identification of biological markers for targeted therapy continues to be a high priority in human cancer treatment [4]. Anti-epidermal growth factor receptor (EGFR) monoclonal antibody treatment, including that with cetuximab and panitumumab, has been reported to improve progression-free survival in advanced CRC patients with wild-type KRAS [5–7]. Despite the undeniable therapeutic progress, a considerable proportion of cancer patients respond poorly to therapy, and therapeutic response cannot be exactly predicted. Therefore, it is of great interest to identify molecular biomarkers for predicting outcome, therapeutic response, and potential therapeutic targets in CRC patients.

The human epidermal growth factor receptor 2 (HER2) is a transmembrane receptor tyrosine kinase, which is a member of the EGFR family [8,9]. Activation of HER2 plays a key role in cell proliferation, cell differentiation, inhibition of apoptosis, and tumor progression [8–10]. Trastuzumab, a humanized monoclonal antibody, targets the extracellular domain of the HER2 receptor. Its therapeutic benefit has been demonstrated in HER2-positive breast cancer patients [11,12]. In addition, one landmark study in 2010 reported that the combination of trastuzumab with conventional chemotherapy significantly improved patients’ survival in patients with HER2-positive advanced gastric or gastroesophageal junction cancer [13]. HER2 gene amplification and/or protein overexpression were observed in 15–20% of breast cancer cases and were correlated with aggressive phenotype, metastases, and adverse clinical outcome [14,15]. On the other hand, HER2 gene amplification and/or protein overexpression were detected in...
−22% of gastric cancer patients, but prognostic significance was controversial [16]. Because trastuzumab has significantly improved overall survival in breast and gastric cancers, it is of great clinical interest whether HER2 blockade may be a useful clinical strategy in other human cancers [17]. Although a few studies have reported the incidence and clinical implication of HER2 status in CRC patients [18–22], its clinical significance has not yet been fully elucidated.

We, therefore, conducted this study to determine the incidence and clinical significance of HER2-positive status in consecutive primary CRC patients. Because targeted chemotherapy was applied to advanced CRC patients in daily practice and the advanced cases were not sufficiently included in consecutive cohort 1, we additionally enrolled advanced CRC patients with synchronous or metachronous distant metastasis (cohort 2). We evaluated HER2 gene amplification, messenger RNA (mRNA) transcription, and protein expression status in the primary tumors of two retrospective cohorts, and also analyzed the concordance rates of the detection methods.

Materials and Methods

Patients and samples
A total of 539 CRC cases treated by radical surgery without any preoperative therapy were enrolled into the study from the Department of Pathology, Seoul National University Bundang Hospital. Cohort 1 consisted of 365 consecutive CRC patients treated between January 2005 and December 2006. Cohort 2 comprised of 174 advanced CRC patients with synchronous or metachronous distant metastasis, who had undergone surgical resections for primary CRC between May 2003 and December 2009 except cohort 1. All the patients received treatment according to standard practice guidelines following surgery, unless medically contraindicated. All the cases were reviewed by 2 pathologists (A.N.S and H.S.L) and were staged according to the 7th edition of the American Joint Committee’s Cancer Staging Manual [23]. Clinicopathological characteristics were obtained from patients’ medical records and pathology reports. Follow-up information including the patient outcome and the time interval between the date of surgical resection and death was collected. The cases lost to follow-up and deaths from causes other than CRC were considered censored data for the survival analysis.

Ethical statement
All human specimens were obtained from the files of surgically resected cases examined at the Department of Pathology, Seoul National University Bundang Hospital for the pathologic diagnosis. The retrospective study was performed using the stored samples after the pathologic diagnosis, and all of the samples were anonymized before the study. The study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (reference: B-1210/174-301). The participants did not provide written informed consent in this study. The Institutional Review Board waived the need for written informed consent under the condition of anonymization and no additional intervention to the participants.

Tissue array method
For both cohorts, surgically resected primary CRC specimens were formalin-fixed and paraffin-embedded (FFPE). These primary tumor blocks were used for the construction of tissue array blocks. Briefly, from the representative areas of the harvested blocks in each case, a single core with a diameter of 2 mm were obtained and precisely arranged into new recipient blocks using a trephine apparatus, as described previously by the author’s group (Superbiochips Laboratories, Seoul, South Korea) [24]. An adequate case was defined as a tumor occupying ≥20% of the core area.

Immunohistochemistry (IHC)
HER2 IHC was performed using PATHWAY anti-HER2/ncu (4B3; rabbit monoclonal; pre-dilution; Ventana Medical Systems, Tucson, AZ, USA) antibody and ultraView Universal DAB kit (Ventana Medical Systems) on an automated immunostainer (BenchMark XT; Ventana Medical Systems), according to the manufacturer’s instructions. IHC scoring was independently performed by two pathologists (A.N.S and H.S.L) without prior knowledge of clinicopathological information or molecular results obtained via other methods. The scoring was performed according to the DAKO HercepTest™ guidelines (DAKO) for gastric cancer as follows [25]: 0, no reactivity or membrane staining in <10% of tumor cells; 1+, faint/barely perceptible partial membrane staining in ≥10% of tumor cells; 2+, weak-to-moderate complete or basolateral membrane staining in ≥10% of tumor cells; 3+, moderate-to-strong complete membrane staining in ≥10% of tumor cells. The two pathologists completely agreed on all the IHC 3+ cases, whereas final consensus was determined by discussion via the multi-head microscope in a few discrepant cases with IHC 1+ or 2+. IHC 2+ and 3+ were considered to indicate protein overexpression.

HER2 mRNA in situ hybridization (ISH)
For detecting HER2 mRNA overtranscription, RNAscope 2-plex (Advanced Cell Diagnostics, Hayward, CA, USA) was performed according to the manufacturer’s standard recommendations. The interpretation was performed according to the instructions in the RNA scope FFPE Assay Kit as described previously [26]: no staining (score of 0); staining in <10% of tumor cells that was difficult to identify at x40 objective lens (score of 1); staining in ≥10% of tumor cells that was difficult to identify at x20 objective lens but easy at x40 objective lens (score of 2); staining in ≥10% of tumor cells that was difficult to identify at x10 objective lens but easy at x20 objective lens (score of 3); staining in ≥10% of tumor cells that was easy to identify at x10 objective lens (score of 4). A score of 4 indicates HER2 overtranscription.ISH was independently interpreted by two pathologists (A.N.S and H.S.L) without prior knowledge of clinicopathological information or HER2 status obtained via other methods.

Dual-color silver in-situ hybridisation (SISH)
Bright-field dual-color SISH analysis was performed using the automatic SISH staining device (BenchMark XT, Ventana Medical Systems), according to the manufacturer’s protocols for INFORM HER2 DNA and INFORM Chromosome 17 (CEP17) probes (Ventana Medical Systems). HER2/CEP17 SISH signals were counted according to the interpretive guideline for Ventana INFORM HER2 DNA probe staining of gastric cancer cells (Ventana Medical Systems). Tumor cells were scanned for hot spots by using 20× or 40× objectives, and the area with the highest signals was selected. The signals were counted in 20 non-overlapping tumor cell nuclei from each case using 60× or 100× objectives by two pathologists (A.N.S and H.S.L) who were blinded to HER2 status by other detection methods and clinical information. Small or large clusters were considered to be 6 signals and 12 signals, respectively. HER2 gene amplification was defined as a HER2/CEP17 ratio of ≥2.0 in 20 tumor nuclei. The equivocal cases (ratio: 1.8 to 2.2) were recounted in at least 20 non-overlapping nuclei of different tumor cells at a second target area,
# Table 1. Demographics and clinical characteristics of patients in each cohort.

| Characteristic                        | Cohort 1 | Cohort 2 |
|---------------------------------------|----------|----------|
|                                       | N (%)    | N (%)    |
| **Age (years)**                       |          |          |
| Median                                | 65.0     | 60.0     |
| Range                                 | 20 to 95 | 28 to 93 |
| **Gender**                            |          |          |
| Male                                  | 202 (55.3)| 94 (54.0)|
| Female                                | 163 (44.7)| 80 (46.0)|
| **Histologic differentiation**        |          |          |
| Low grade                             | 331 (93.5)| 149 (85.6)|
| High grade                            | 23 (6.5) | 25 (14.4) |
| **Primary location**                  |          |          |
| Cecum                                 | 12 (3.3) | 8 (4.6)  |
| Ascending                              | 54 (14.8)| 17 (9.8) |
| Hepatic flexure                       | 20 (5.5) | 13 (7.3) |
| Transverse                            | 16 (4.4) | 9 (5.2)  |
| Splenic flexure                       | 6 (1.6)  | 5 (2.9)  |
| Descending                            | 18 (4.9) | 8 (4.6)  |
| Sigmoid                               | 114 (31.2)| 47 (27.0)|
| Rectum                                | 125 (34.2)| 67 (38.5)|
| **Tumor border**                      |          |          |
| Expanding                              | 59 (16.2)| 15 (8.6) |
| Infiltrative                          | 306 (83.8)| 159 (91.4)|
| **Tumor size (cm)**                   |          |          |
| Median                                | 5.0      | 5.4      |
| Range                                 | 1.0 to 13.0| 2.0 to 27.0|
| **Tumor depth (pT)**                  |          |          |
| 1                                     | 14 (3.8) | 1 (0.6)  |
| 2                                     | 46 (12.6)| 4 (2.3)  |
| 3                                     | 238 (65.2)| 102 (58.6)|
| 4                                     | 67 (18.4)| 67 (38.5)|
| **LN metastasis**                     |          |          |
| Absent                                | 170 (46.6)| 32 (18.4)|
| Present                               | 195 (53.4)| 142 (81.6)|
| Distant metastasis at initial diagnosis|          |          |
| Absent                                | 299 (81.9)| 61 (35.1)|
| Present                               | 66 (18.1) | 113 (64.9)|
| **TNM stage**                         |          |          |
| I                                     | 46 (12.6)| 4 (2.3)  |
| II                                    | 118 (32.3)| 17 (9.8) |
| III                                   | 135 (37.0)| 40 (23.0)|
| IV                                    | 66 (18.1)| 113 (64.9)|
| **Microsatellite instability (MSI)**  |          |          |
| MSS/MSI-L                             | 321 (90.9)| 159 (98.1)|
| MSI-H                                 | 32 (9.1)  | 3 (1.9)  |
| Total                                 | 365 (100.0)| 174 (100.0)|

Abbreviations: N, number; LN, lymph node; TNM, tumor-node-metastasis; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.

*Missing value was included.

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and a new \( \text{HER2} / \text{CEP17} \) ratio was recalculated. Normal colon epithelial cells and other adjacent benign cells served as the internal controls.

**Microsatellite instability (MSI)**

MSI results were generated by comparison of the allelic profiles of the 5 microsatellite markers (BAT-26, BAT-25, D5S346, D17S250, and S2S123) in tumor and corresponding normal samples. The polymerase chain reaction products for FFPE tissues were analyzed using a DNA auto-sequence (ABI 3731 genetic analyzer; Applied Biosystems, Foster City, CA) according to the protocol described previously [27].

**Statistical analyses**

The association between clinicopathological features and \( \text{HER2} \) status was analysed by using Chi-square or Fisher's exact test, and Wilcoxon/Mann-Whitney test, if appropriate. The Spearman rank correlation or Kendall's tau-b test was used to assess the correlation between the detection methods. The association between \( \text{HER2} \) status and overall survival was determined using the Kaplan-Meier method, and the significance of the differences between groups was compared using the log-rank test or Breslow test, if survival curves were crossed during follow-up periods. Multivariate survival analyses were performed using the Cox proportional hazards model, and the hazard ratio and its 95% confidence interval were calculated for each factor. All the tests were two-sided, with \( P \) value of <0.05 considered to indicate statistical significance. All the statistical analyses were performed using the IBM SPSS statistics 20 (Armonk, NY, USA) software.

**Results**

**Patients’ clinicopathological characteristics**

Table 1 provides demographics and baseline clinicopathological characteristics of the patients included in each cohort. Cohort 1 included 202 (55.3%) male and 163 (44.7%) female with median age of 65 years (range: 20–95 years). Additionally, among 353 patients with MSI analysis available, 321 (90.9%) had microsatellite stable (MSS) or microsatellite instability-low (MSI-L) whereas 32 (9.1%) had microsatellite instability-high (MSI-H). On the other hand, cohort 2 included 94 (54.0%) male and 80 (46.0%) female with median age of 60 years (range: 28–93 years). MSS/MSI-L was found in 159 (98.1%) and MSI-H in 3 (1.9%) out of 162 patients with MSI analysis available.

**\( \text{HER2} \) status in CRC patients**

The \( \text{HER2} \) protein was faintly or weakly expressed in the membrane and/or cytoplasm of normal colonic epithelium, whereas the membrane of the tumor cells presented various staining patterns (Figure 1). In consecutive cohort 1, IHC 0 was found in 296 of 365 patients (81.1%), IHC 1+ in 47 (12.9%), IHC 2+ in 14 (3.8%), and IHC 3+ in 8 (2.2%). In cohort 2 of advanced cases, IHC 0 was observed in 139 of 174 patients (79.9%), IHC 1+ in 25 (14.4%), IHC 2+ in 5 (2.9%), and IHC 3+ in 5 (2.9%). \( \text{HER2} \) overexpression was detected in 6.0% of cohort 1 and 5.8% of cohort 2.

A few scattered brown punctate dots were observed in the nucleus and/or cytoplasm of normal colonic epithelium by using \( \text{HER2} \) mRNA ISH, whereas various nuclear and/or cytoplasmic staining was observed in the tumor cells (Figure 1). In cohort 1, ISH score 0 was observed in 12 of 365 patients (3.3%), score 1 in 118 (32.3%), score 2 in 160 (43.8%), score 3 in 66 (18.1%), and score 4 in 9 (2.5%). In cohort 2, ISH score 0 was observed in 11 of 174 patients (6.3%), score 1 in 41 (23.6%), score 2 in 93 (53.4%), score 3 in 25 (14.4%), and score 4 in 4 (2.3%). \( \text{HER2} \) mRNA overtranscription was observed in 2.5% of cohort 1 and 2.3% of cohort 2.

\( \text{HER2} \) signals were detected as disomy in normal colonic epithelium, thus it was used as the internal controls. However, \( \text{HER2} \) signals varied throughout the tumor cells (Figure 1). The median \( \text{HER2} / \text{CEP17} \) ratio was 1.16 (range: 0.68 to 19.62) in cohort 1 and 1.19 (range: 0.57 to 21.89) in cohort 2. \( \text{HER2} \) gene amplification was detected in 21 of 365 patients (5.8%) in cohort 1, and 11 of 174 (6.3%) in cohort 2. There was no significant difference in the incidence of \( \text{HER2} \) positivity between consecutive cohort 1 cases and advanced cohort 2 cases, regardless of the detection method (IHC, ISH, or SISH). Of a total of 32 amplified cases, mean \( \text{HER2} \) gene copy numbers (GCN) was 6 or more in 16 cases. According to the gastric cancer criteria set by the ToGA trial [13], \( \text{HER2} \) positivity is defined as IHC 3+ or IHC 2+ with \( \text{HER2} \) gene amplification. \( \text{HER2} \) positivity was found in 3.6% (13 of 365 patients) of cohort 1 and 4.0% (7 of 174 patients) of cohort 2. We also investigated chromosome 17 (CEP17) alterations and observed a homogeneous CEP17 signal pattern.
### Table 2. The correlation between immunohistochemistry and silver in situ hybridization for HER2 in all CRCs of combined cohort.

| HER2 | Immunohistochemistry score | mRNA in situ hybridization score |
|------|---------------------------|----------------------------------|
|      | 3+ | 2+ | 1+ | 0 | Total | 4 | 3 | 2 | 1 & 0 | Total |
|      | N (%) | N (%) | N (%) | N (%) | N (%) | N (%) | N (%) | N (%) | N (%) | N (%) |
| SISH + | 13 (100.0) | 7 (36.8) | 7 (9.7) | 5 (1.1) | 32 (5.9) | 13 (100.0) | 8 (8.8) | 7 (2.8) | 4 (2.2) | 32 (5.9) |
| SISH - | 0 (0) | 12 (63.2) | 65 (90.3) | 430 (98.9) | 507 (94.1) | 0 (0) | 83 (91.2) | 246 (97.2) | 178 (97.8) | 507 (94.1) |
| Total | 13 (100.0) | 19 (100.0) | 73 (100.0) | 435 (100.0) | 539 (100.0) | 13 (100.0) | 91 (100.0) | 253 (100.0) | 182 (100.0) | 539 (100.0) |

Abbreviations: CRC, colorectal cancer; HER2, human epidermal growth factor receptor 2; SISH, silver in-situ hybridization; N, number; mRNA, messenger RNA.

All the IHC + cases and all the ISH score 4 cases showed HER2 gene amplification by dual-color SISH. The concordance rate was 95.5% between dual-color SISH and IHC detection methods. doi:10.1371/journal.pone.0098528.t002
Figure 2. Representative figures of the cases with gene amplification and mRNA overtranscription, but without HER2 protein overexpression. (A–C) HER2 IHC + in cancer focal area (A), focal mRNA overtranscription (B) and HER2 gene amplification (C) in accordance with protein expression area. (D–F) No expression of HER2 protein (D), but diffuse mRNA overtranscription (E) and HER2 gene amplification (F) in one case. doi:10.1371/journal.pone.0098528.g002

Discussion

The purpose of this study was to identify the incidence of HER2-positive status in two retrospective cohorts, including consecutive CRCs and advanced CRCs with distant metastasis, and to clarify their clinical significance. Herein, we observed that HER2 gene amplification and protein overexpression were found in about 6% of CRCs from both cohorts, CRCs with HER2 gene amplification were located more commonly in the rectum than in the right or left colon, HER2 status was not associated with aggressive clinicopathological features or worse prognosis, and the concordance rates between detection methods were very high. To the best of our knowledge, this is the first report to evaluate HER2-positive status using several techniques in a large-scale study of East Asian CRC patients.

To date, several studies have reported that the frequency of HER2 protein overexpression varies widely, from 0% to 80% in CRC [28,29], and its prognostic significance is controversial [28,30–32]. This debate might be attributed by several causes, such as primary antibody difference, a difference in scoring systems for HER2 protein expression, a difference of technical approach, sample size, racial differences, and heterogeneity of study population. When accepted staining and scoring techniques were used, the rate of membranous and cytoplasmic overexpression for HER2 were reported in approximately 5% and 30%, respectively [29]. We used accepted staining and scoring methods, and membranous overexpression of HER2 was observed in approximately 6%. These findings suggest that trastuzumab may be effective in a minority of CRC patients [33]. Alternatively, intracellular HER2-targeting compounds might be attractive treatment option in one-third of CRC patients, if cytoplasmic HER2 is really actively involved in carcinogenesis of CRC [29,33].

It is worth noting that the concordance rate was 95.5% between dual-color SISH and IHC detection methods in the present study. When excluding equivocally immunostained cases (IHC 2+), the concordance rate was 97.7%. These concordance rates were enough to use just the IHC method for screening HER2 gene amplification, and similar to the rates seen in gastro-esophageal cancer in the previous studies [4,34,35]. Among the discrepant cases, one case had HER2 gene amplification with homogeneous distribution, but HER2 protein was not expressed. This finding has been reported in gastro-esophageal cancer accounting for 2% to 33% of the cases [4,34–37]. This phenomenon necessitates further studies, but the following hypothesis could explain it: erroneous post-translation processes, such as incorrect three dimensional folding, dysfunctional localization of the protein to the cell membrane, and inappropriate protein glycosylation, leading to decreased HER2 protein expression, even though the HER2 gene is amplified [4]. In our case, HER2 mRNA was overtranscribed with homogenous distribution supporting the hypothesis noted above. However, despite high concordance rates between detection methods in the present study, HER2 mRNA ISH is currently not a standard method for assessment of HER2 status, and there has not yet been established cut-off to define mRNA overtranscription. Accordingly, further studies are necessary to validate HER2 mRNA ISH method.

Recently, Conradi et al. [21] screened for HER2 positivity using the same methods and criteria as ours, and reported that HER2 positivity was observed in 12.4% of rectal biopsy samples and 26.7% of rectal resected specimen. Furthermore, HER2 positivity in resected specimens independently correlated with prolonged cancer-specific survival in rectal cancer patients. Schafani et al. [22] also evaluated for HER2 positivity in high-risk, locally advanced rectal cancer patients in the EXPERT-C trial of neoadjuvant capcitabine and oxaliplatin and chemoradiotherapy (CRT) with or without cetuximab. However, in their study, HER2 positivity was only 4.3%, and it had no association with clinicopathologic parameters and patient outcome. Our study comprised right and left colon cancers and rectal cancers; thus, we were able to analyse the frequency of HER2 amplification and overexpression according to primary location. HER2 amplification was most frequently found in rectal cancer than in any other primary site, and HER2 positivity of rectal cancer was 8.0% in cohort 1 and 7.5% in cohort 2. The inconsistency for frequency of HER2 positivity likely reflects the possible effect of several factors such as ethnicity, different study population, and tumor heterogeneity. Especially, in two previous studies, disagreement of HER2 positivity between biopsy and resected specimen was observed [21,22]. In light of these findings, it is suggested that intratumoral HER2 heterogeneity may exist in CRCs, which has also been reported in studies of gastric cancer [10]. Further studies are required for clarification.

Traditionally, CEP17 alterations have affected the measurement of the HER2/CEP17 ratio. Consequently some cases were misclassified as non-amplified. In the present study, CEP17
### Table 3. The relationship between tumor location and *HER2* status in each cohort.

| *HER2* status | Primary location (cohort 1) | Primary location (cohort 2) | \( \rho \) |
|---------------|----------------------------|----------------------------|---------|
|               | Right colon | Left colon | Rectum | Right colon | Left colon | Rectum | Right colon | Left colon | Rectum |\( \rho \) |
| *HER2* IHC    |             |             |        |             |             |        |             |             |        |        |
| IHC 0/1+      | N (97.1)    | N (94.9)    | N (90.4)| 45 (95.7)   | 59 (98.3)  | 60 (89.6)| 0.033       |             |        |        |
| IHC 2+/3+     | 3 (2.9)     | 7 (5.1)     | 12 (9.6)| 2 (4.3)     | 1 (1.7)    | 7 (10.4)| 0.119       |             |        |        |
| *HER2* ISH    |             |             |        |             |             |        |             |             |        |        |
| score 0-3     | 102 (100)   | 137 (99.3)  | 117 (93.6)| 47 (100)   | 60 (100)  | 63 (94.0)| 0.001       |             |        | 0.026   |
| score 4       | 0 (0)       | 1 (0.7)     | 8 (6.4) | 0 (0)       | 0 (0)     | 4 (6.0)| 0.011       |             |        |        |
| *HER2* SISH   |             |             |        |             |             |        |             |             |        |        |
| not amplified | 99 (97.1)   | 133 (96.4)  | 112 (89.6)| 46 (97.9)  | 59 (98.3) | 58 (86.6)| 0.013       |             |        | 0.009   |
| amplified     | 3 (2.9)     | 5 (3.6)     | 13 (10.4)| 1 (2.1)    | 1 (1.7)   | 9 (13.4)| 0.001       |             |        |        |
| *HER2* positivity* |         |             |        |             |             |        |             |             |        |        |
| negative      | 102 (100)   | 135 (97.8)  | 115 (92.0)| 46 (97.9)  | 59 (98.3) | 62 (92.5)| 0.001       |             |        | 0.125   |
| positive      | 0 (0)       | 3 (2.2)     | 10 (8.0) | 1 (2.1)    | 1 (1.7)   | 5 (7.5)| 0.011       |             |        |        |
| Total         | 102 (27.9)  | 138 (37.8)  | 125 (34.2)| 47 (27.0)  | 60 (34.5) | 67 (38.5)| 0.013       |             |        |        |

Abbreviations: *HER2*, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in-situ hybridization; SISH, silver in-situ hybridization; N, number.

*HER2* positivity was defined as *HER2* IHC 3+ and *HER2* IHC 2+ with gene amplification.

*P* values were estimated using linear-by-linear association.

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polyosomy was a rare event accounting for 2.6% of the cases, thus it did not induce misleading interpretation in HER2 gene amplification. CEP17 polyosomy was not clinically significant in patients with CRCs.

Although two clinical trials had tried to investigate the benefit of anti-HER2 therapy in advanced or metastatic CRC, the trials were closed prematurely due to low accrual related to the low incidence of HER2 overexpression in advanced CRC patients [38,39]. However, one recent study showed that HER2 served to predict resistance to anti-EGFR monoclonal antibody in patient-derived xenografts from metastatic CRC, leading the authors to suggest a possibility of combined therapies in cetuximab-resistant CRC patients [40]. Moreover, a recent HERACLES trial (HER2 Amplification for Colo-rectaL Cancer Enhanced Stratification) is currently investigating the use of trastuzumab plus lapatinib or pertuzumab in metastatic CRCs with HER2 amplification [41]. Therefore, our findings would be helpful in developing and applying HER2 targeted therapy in CRC patients. Because mutation results of KRAS and BRAF were not included in the present study, we were unable to elucidate relationship between HER2 status and KRAS or BRAF in CRC. It is the concerned limitation of our study since KRAS and BRAF are very important to determine treatment approach in CRC patients. Additionally, our study has potential weaknesses, such as retrospective designed study, assessment of HER2 using a tissue array method, heterogenous study population in a single institution, and selecting patients in cohort 2. We enrolled the CRC patients with available surgically resected cancer tissues from primary tumors in cohort 2. Not all advanced CRC patients with metastatic diseases were included and far advanced cases were not enrolled because of their inoperability. Therefore, unrecognized biases might have influenced our survival results. Furthermore, a small number of HER2 positive tumors could preclude robust statistical analysis, thus further large-scale studies are required to validate our results.

In conclusion, HER2 gene amplification and protein overexpression were identified in about 6% of CRC patients and has no clinical implication except in tumor location in the present study. We also demonstrated high concordance rates between IHC and dual-color SISH methods in the combined cohorts. Although a minority of CRC patients exhibited HER2 gene amplification, these patients would be potential candidates for anti-HER2 therapy, and IHC could be primary screening test for patient selection.

Supporting Information

Figure S1 Kaplan-Meier survival curves according to HER2 status by each detection method. (A–B) Survival curves according to HER2 protein expression status in cohort 1 (A) and cohort 2 (B). (C–D) Survival curves according to HER2 mRNA expression status in cohort 1 (C) and cohort 2 (D). (E–F) Survival curves according to HER2 gene amplification status in cohort 1 (E) and cohort 2 (F).

Table S1 The relationship between immunohistochemistry and mRNA in situ hybridization for HER2 in CRCs of combined cohort. (DOCX)

Table S2 The details of the cases with HER2 gene amplification, but IHC 0 or 1+ results. (DOCX)

Table S3 The association between HER2 protein expression and clinicopathologic variables in CRCs of each cohort. (DOCX)

Table S4 The association between HER2 gene amplification and clinicopathologic factors in CRCs of each cohort. (DOCX)

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Author Contributions

Conceived and designed the experiments: ANS YK WHK HSL. Performed the experiments: ANS YK WHK HSL. Analyzed the data: ANS YK GC HSL. Contributed reagents/materials/analysis tools: ANS YK DWK SBK GC WHK HSL. Wrote the paper: ANS HSL. Final approval of the version to be published: HSL.

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