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Utility of comprehensive genomic sequencing for detecting HER2-positive colorectal cancer

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Running title

CGS and HER2-positive CRC

Conflicts of interest

All authors have no conflicts of interest regarding this manuscript.

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Keywords: HER2, colorectal cancer, next-generation sequencing, comprehensive genomic sequencing, immunohistochemistry, fluorescence *in situ* hybridization

Abstract

HER2-targeted therapy is considered effective for *KRAS* codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (CRC). In general, HER2 status is determined by the use of immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH).

Comprehensive genomic sequencing (CGS) enables the detection of gene mutations and copy number alterations including *KRAS* mutation and *HER2* amplification; however, little is known about the utility of CGS for detecting HER2-positive CRC. To assess its utility, we retrospectively investigated 201 patients with stage I–IV CRC. The HER2 status of the primary site was assessed using IHC and FISH, and *HER2* amplification of the primary site was also assessed using CGS, and the findings of these approaches were compared in each patient. CGS successfully detected alterations in 415 genes including *KRAS* codon 12/13 mutation and *HER2* amplification. Fifty-nine (29%) patients had a *KRAS* codon 12/13 mutation. Ten (5%) patients were diagnosed as HER2-positive because of HER2 IHC 3+, and the same 10 (5%) patients had *HER2* amplification evaluated using CGS. The results of HER2 status and *HER2* amplification were completely identical in all 201 patients ($P < 0.001$). Nine of the 10 HER2-positive patients were *KRAS* 12/13 wild-type and were considered possible candidates for HER2-targeted therapy. CGS has the same utility as IHC and FISH for detecting HER2-positive patients who are candidates for HER2-targeted therapy, and facilitates precision medicine and tailor-made treatment.

Word count of the abstract: 226 words

Highlights

- HER2-targeted therapy is possibly effective for HER2-positive colorectal cancer.
- Generally, HER2 status is determined by immunohistochemistry (IHC).
- *HER2* amplification can be also detected by comprehensive genomic sequencing (CGS).
- This study showed that the results of IHC and CGS were completely identical.
- CGS has the same utility as IHC for detecting HER2-positive colorectal cancer.

Introduction

HER2, a member of the human epidermal growth factor receptor (HER) family, is a plasma membrane protein with intrinsic tyrosine kinase activity [1]. In contrast to other HER family members, HER2 is a receptor with no ligand; HER2 protein activation depends directly on its dimerization with another HER2 monomer or with a monomer of another HER family member, such as HER3 [2, 3]. After dimerization, the most important signaling pathways, such as the mitogen-activated protein kinase (MAPK) and PI3K pathways, are activated by HER2, resulting in cell duplication and regulation of apoptosis [4]. HER2 signal transduction can be dysregulated via different mechanisms. HER2 overexpression, generally resulting from *HER2* gene amplification [5, 6], is one of the main causes of the constitutive activation of HER2 signal transduction in many cancers [7].

HER2 overexpression and/or amplification are associated with carcinogenesis, poor prognosis, and may also predict the response to chemotherapy [7]. Under the rationale for using anti-HER2 targeted approaches to block its oncogenic effects, anti-HER2 monoclonal antibodies have been designed for HER2-positive tumors. Trastuzumab is a monoclonal antibody that recognizes the extracellular portion of the HER2 receptor, and once bound, it completely inhibits HER2 activity [8]. Today, trastuzumab treatment is the standard care for HER2-positive breast [9] and gastric cancers [10]. In addition to breast and gastric cancers, preclinical models and clinical trials are underway to assess the efficacy of HER2-targeted therapy for patients with colorectal cancer (CRC) [11]. Several studies reported the incidence of HER2-positive cases and its clinical significance in CRC [12-19], and the use of trastuzumab for HER2-positive cases is recognized as a promising treatment strategy for

KRAS wild-type metastatic CRC [19-21]. Recently, the HERACLES trial demonstrated the efficacy of dual-targeted therapy with trastuzumab and lapatinib for *KRAS* codon 12/13 wild-type and HER2-positive CRC [22]. Hence, both *KRAS* mutation and HER2 status should be evaluated to select candidates for HER2-targeted therapy in CRC.

In current histopathology, the HER2 status of breast and gastric cancer is determined by immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) [23, 24]; likewise, the HER2 status of CRC is usually evaluated by IHC and FISH [22]. Conversely, by utilizing next-generation sequencing technology, projects such as The Cancer Genome Atlas (TCGA) have profiled genomic changes in several cancers including CRC [25]. Comprehensive genomic sequencing (CGS) can detect gene mutations and copy number alterations in a single assay, including *KRAS* mutation and *HER2* amplification. However, it is not clear whether CGS has the same diagnostic value as IHC and FISH for detecting HER2-positive CRC. Therefore, the aim of this study was to clarify the utility of CGS for detecting HER2-positive CRC.

Materials and methods

Patients

A total of 201 patients diagnosed with stage I - IV CRC according to AJCC 7th edition [26] who performed primary tumor resection between 2009 and 2015 at Niigata University Medical and Dental Hospital or Niigata Cancer Center Hospital were enrolled. Patients with familial adenomatous polyposis or inflammatory bowel disease were excluded. None of the patients had received neoadjuvant radiation or HER2-targeted therapy. This retrospective

study was performed in accordance with the Helsinki Declaration, and the Ethics Committee of the School of Medicine, Niigata University approved the study protocol.

Pathological evaluation of HER2 status

Resected specimens were fixed immediately in 10% buffered formalin for 24 h, and the entire tumor was cut into step-wise sections and embedded in paraffin. Each section was examined with hematoxylin and eosin (HE) staining, and the archives were reviewed for each case to select one cross-section with the most invasion. The corresponding paraffin blocks were re-cut, making 4- μ m thick slices, and 3 serial sections were assigned for HE staining, anti-HER2 staining, and a negative control. HER2 status was determined according to the HERACLES diagnostic criteria: HER2-positive status was defined as tumors with a 3+ HER2 score in more than 50% of cells by IHC or with a 2+ HER2 score and a HER2:CEP17 ratio higher than 2.0 in more than 50% of cells by FISH [22].

IHC for evaluating HER2 status

Specimen slides were deparaffinized, followed by dehydration with ethanol. The sections were washed 3 times with phosphate-buffered saline for 5 min. The sections were digested with 0.03 U/g protease, diluted with 0.05 M Tris-HCl pH 7.6, at room temperature for 5 min (protease: type EX No. P3111; SIGMA Aldrich Corp., St. Louis, MO). After blocking nonspecific reactivity with 3% H₂O₂ for 5 min, an anti-HER2 monoclonal antibody (SV2-

61γ; Nichirei Biosciences, Inc., Tokyo, Japan) was applied at 5 mg/mL (diluted with 0.5% bovine serum albumin/phosphate-buffered saline) at room temperature for 30 min. Then, the sections were incubated with Histofine Simple Stain MAX PO (M) (Nichirei Biosciences, Inc.) at room temperature for 30 min, followed by incubation for 10 min with a DAB kit (Nichirei Biosciences, Inc.), and the sections were counterstained with hematoxylin. Positive tissue controls were included in each run; these tissues were breast tumors with known HER2 protein overexpression and *HER2* gene amplification [27].

FISH for evaluating HER2 status

Specimen slides were deparaffinized, followed by dehydration with ethanol. Each slide was treated with a Vysis Paraffin Pretreatment Kit (Abbott Molecular, Des Plaines, IL). After pretreatment, the resulting specimen DNA was denatured and the slides were dehydrated in serial ethanol solutions and subsequently allowed to hybridize with the PathVysion (Abbott Molecular) probe mixture. Following hybridization, excess and unbound probe was removed by a series of washes, and the chromosomes and nuclei were counterstained with the DNA-specific stain 4',6 diamidino-2-phenylindole, which fluoresces blue under UV illumination. Hybridization of the HER2/neu and CEP 17 DNA probes was viewed using a fluorescence microscope equipped with appropriate excitation and emission filters, allowing visualization of the orange and green fluorescent signals and the blue counterstained chromosomes and nuclei [28].

CGS of primary sites

Archival tissue in the form of formalin-fixed, paraffin-embedded tumor or unstained tissue sections obtained during primary tumor resection were used for CGS, and *HER2* amplification, *HER2* mutation, and *KRAS* exon 2 (codon 12 and 13) mutation were evaluated. An independent pathologist evaluated tumor content on HE-stained slides for each study sample to ensure >50% tumor content was present. Where applicable, unstained slides were macro-dissected to enrich for tumor content and DNA was extracted using a BioStic FFPE Tissue DNA Isolation Kit (Mo Bio Laboratories, Inc., CA). All sample preparation, CGS, and analytics were performed in a CLIA/CAP-accredited laboratory (KEW, Cambridge, MA). DNA (50–150 ng) fragment libraries were prepared and enriched for the 415 gene panel with CancerPlex (KEW, Cambridge, MA) [29]. CancerPlex is a clinically validated 415 gene panel enriched for coding regions and selected introns of genes with a known association to cancer. Sequencing was performed on the Illumina MiSeq and NextSeq platforms with an average 500× sequencing depth. Genomic data were then processed through a proprietary bioinformatics platform and knowledgebase to identify multiple classes of genomic abnormalities including single nucleotide substitutions (SNPs), small insertions/deletions (indels), copy number variation, and translocations. A threshold of 10% allelic fraction was used for SNPs and indels and thresholds of >2.5-fold (gain) and 0.5-fold (loss) were used.

HER2 status and other clinicopathological characteristics

To analyze the relationship between *HER2* status and other clinicopathological characteristics, 11 clinicopathological variables were examined in all 201 patients: age (<65 vs. ≥65 years), sex (male vs. female), tumor location (right vs. left), tumor size (<50 vs. ≥50 mm), T category (T1, 2 vs. T3, 4), histopathological grading (G1, 2 vs. G3), lymphatic invasion (absence vs. presence), venous invasion (absence vs. presence), N category (N0 vs.

N1, 2), M category (M1a vs. M1b), *KRAS* exon 2 (codons 12 and 13) mutation (wild-type vs. mutant), *NRAS* exon 2 (codons 12 and 13) mutation (wild-type vs. mutant), and *BRAF* V600E mutation (wild-type vs. mutant). Histopathological markers associated with deficiency of mismatch repair gene, such as medullary type, mucinous type, Crohn-like lymphoid reaction, and tumor infiltrating lymphocytes were analyzed by reported method [30]. A total of 110 of 201 patients were randomly selected and evaluated for MutL homologue 1 (MLH1)/MutS homologue 2 (MSH2) status using immunohistochemical stainings. The primary antibodies were MLH1 (1:50; BD Biosciences PharMingen, San Diego, CA) and MSH2 (1:50; Leica Microbiosystems, Tokyo, Japan).

Comparison of HER2 status between primary and paired metastatic sites

In the present study, 45 of the 201 patients underwent metastasectomy. The following metastatic sites were evaluated for HER2 status by the same methods as for the primary site [22]: liver (30 patients), lung (4 patients), ovary (3 patients), peritoneum (2 patients), brain (2 patients), omentum (2 patients), para-aortic lymph node (1 patient), and bone (1 patient).

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 22 (IBM Japan, Inc., Tokyo, Japan). Fisher's exact test was used to evaluate the associations between HER2 status and *HER2* amplification evaluated using CGS, and HER2 status and *HER2* mutation evaluated using CGS. HER2 status and each clinicopathological variable, and HER2 status between the primary site and metastatic site was also evaluated by Fisher's exact test. *P*-values less than 0.05 were considered statistically significant.

Results

IHC and FISH for HER2 status at the primary site

Ten patients were HER2 IHC 3+ in more than 50% of the cancer area (Fig. 1A, B), and 2 patients were HER2 IHC 3+ in less than 50% (Fig. 1C, D). Fifteen patients were HER2 IHC 2+; however, there was no patient with IHC 2+ and a HER2:CEP17 ratio higher than 2.0 in more than 50% of cells by FISH. Hence, 10 of the 201 (5%) patients with HER2 IHC 3+ in more than 50% of the cancer area were diagnosed as HER2-positive by the HERACLES diagnostic criteria.

Association between HER2 status and HER2 amplification evaluated using CGS

CGS successfully detected alterations in 415 genes including *KRAS* codon 12/13 mutation and *HER2* amplification. Forty-nine of the 201 (29%) patients had a *KRAS* codon 12/13 mutation. Ten of the 201 (5%) patients had a *HER2* amplification. The result of *HER2* amplification evaluated using CGS and that of *HER2* status evaluated using IHC were completely identical in all 201 patients ($P < 0.001$) (Table 1) (Table 2). Nine of the 10 *HER2*-positive patients were *KRAS* 12/13 wild-type, who were considered to be possible candidates for *HER2*-targeted therapy (Fig. 2).

Association between HER2 status and HER2 mutation evaluated using CGS

CGS also detected *HER2* mutations (V308M, S310Y, R647K, R678Q, and A879delinsAE) in

6 of 201 (3%) patients. Among the 6 patients, only 1 patient had both *HER2* mutation (S310Y) and *HER2* amplification, and the patient showed HER2 positive (IHC 3+) (Table 2).

No significant association was observed between HER2 status and *HER2* mutation ($P = 0.267$).

HER2 status and other clinicopathological characteristics

There was no significant association between HER2 status and other clinicopathological characteristics (Table 3).

Comparison of HER2 status between the primary and paired metastatic sites

At the metastatic sites, 3 of 45 (7%) patients were HER2-positive. Comparing between the primary and metastatic sites, 44 of the 45 (98%) patients showed a concordant HER2 status (Table 4). Focusing on the 4 patients with a positive HER2 status at the primary site, 3 of these patients were HER2-positive at the metastatic site (Fig. 3); however, the remaining patient was HER2-negative at the metastatic site: IHC score 3+ (100% area) at the primary site and IHC 0 at the metastatic site (para-aortic lymph node).

Discussion

In the present study, we showed two main results regarding the utility of CGS for detecting HER2-positive CRC patients. First, CGS successfully detected *HER2* amplification in 10 of 201 (5%) patients. Second, the result for *HER2* amplification evaluated using CGS and that for HER2 status evaluated using IHC and FISH were completely identical in all 201 patients.

These results imply that CGS can reliably detect HER2-positive CRC patients, who are candidates for HER2-targeted therapy.

HER2-targeted therapy is considered a promising strategy for *KRAS* wild-type metastatic CRC [19-21], and the evaluation of HER2 status and *KRAS* mutation is necessary to select candidates for HER2-targeted therapy. Recently, the HERACLES trial revealed the efficacy of dual targeted therapy with trastuzumab and lapatinib in treatment-refractory, *KRAS* codon 12/13 wild-type, HER2-positive metastatic CRC [22]. In the trial, HER2 status was evaluated using IHC and FISH, and HER2-positivity was defined using the CRC-specific HERACLES diagnostic criteria [22]. In the present study, we also assessed HER2 status according to the HERACLES diagnostic criteria. Conversely, CGS can detect multiple gene alterations including *KRAS* codon 12/13 mutation and *HER2* amplification in a single assay. However, to date, no study has investigated the concordance of HER2-positivity between HER2 status and *HER2* amplification detected by CGS. In the current study, we tested the utility of CGS for detecting HER2-positive CRC using a 415-gene panel designed for solid tumors.

The ultimate goal of cancer genome profiling is to enable precision medicine, i.e., the tailoring of treatments based on the unique genomic changes of each patient's individual tumor. In CRC, gene mutations in the MAPK pathway, such as *KRAS*, *NRAS*, and *BRAF*, are important benchmarks to decide treatment strategies for patients with metastatic CRC. The National Comprehensive Cancer Network guidelines state that all patients with metastatic CRC should have tumor tissue genotyped for *KRAS*, *NRAS*, and *BRAF* mutations [31]. Patients with any known *KRAS* or *NRAS* mutation should not be treated with EGFR-targeted therapy such as cetuximab and panitumumab [32]. In the present study, we successfully detected alterations in 415 genes including *KRAS*, *NRAS*, and *BRAF* mutations and *HER2*

amplification and mutation, which implies that CGS cannot only detect candidates for EGFR-targeted therapy but can also identify those for HER-2 targeted therapy in a single assay.

TCGA project found that 4% of CRC patients have *HER2* mutations [25]. However, to date, *HER2* mutations in CRC has not been fully studied, and it is an open question as to whether *HER2* mutations are clinically important in CRC. Kavuri et al. reported that *HER2* mutations S310F, L755S, V777L, V842I, and L866M are activating mutations in CRC, and the *HER2* activating mutations may be drug targets for the treatment of CRC [33]. In the present study, we identified 6 of 201 (3%) patients with *HER2* mutations (V308M, S310Y, R647K, R678Q, and A879delinsAE), that require further investigation to determine their oncogenicity.

Among the 6 patients with *HER2* mutations, only 1 patient had both *HER2* mutation and *HER2* amplification, who interestingly showed HER2 positive (IHC3+). We speculate that patients with both *HER2* mutation and *HER2* amplification may be candidates for HER2-targeted therapy when the patients show HER2 positive using IHC/FISH. In addition to *HER2* amplification, future studies might have to focus on the significance of *HER2* mutations in CRC, and clarify whether *HER2* mutations are drug targets for CRC treatment.

In this study, we showed the utility of CGS for detecting HER2-positive patients, who are candidates for HER2-targeted therapy; however, HER2-positive patients do not always demonstrate a favorable response to this treatment approach. Discordant HER2 status between primary and metastatic sites is one possible reason underlying drug resistance to HER2-targeted therapy. In gastric cancer, previous reports revealed that discordant HER2

status between primary and metastatic sites was observed in 5–20% of patients [34]. However, in CRC, this issue has not been investigated fully to date. In this study, we demonstrated that 1 of the 4 (25%) patients with HER2-positive status at the primary site was HER2-negative at the metastatic site, suggesting that HER2 status is not necessarily maintained during the metastatic process. In general, tissue samples from the metastatic site may not always be available. Future studies, such as targeted sequencing analysis of liquid biopsy samples might have the potential to resolve this issue and facilitate precision medicine [35].

Regarding pros and cons of IHC/FISH and CGS for evaluating HER2 status, we consider that IHC/FISH are convenient, widely accepted, and established way to evaluate for breast and gastric cancer. However, diagnostic reproducibility may be sometimes problematic; especially, because of tumor heterogeneity. Conversely, CGS is expensive compared to IHC/FISH, and tested in only specialized laboratories. However, CGS has ability to test numerous gene alterations, which are directly associated with treatment strategy, in a single assay. We believe that CGS has possibility to facilitate precision medicine.

This study has two potential limitations. First, this was a retrospective study performed at two institutions and included a small number of patients. Second, we could not demonstrate an association between *HER2* amplification detected by CGS and the efficacy of HER2-targeted therapy. Future studies should investigate patients with metastatic CRC to determine whether HER2-targeted therapy is effective for patients with *HER2* amplification detected by CGS.

Conclusion

There is a small, distinct category of HER2-positive CRC patients who are candidates for HER2-targeted therapy. CGS has the same sensitivity as IHC and FISH for detecting HER2-positive patients and has the potential to facilitate precision medicine and tailor-made treatment.

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Conflicts of interest

All authors have no conflicts of interest regarding this manuscript.

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Figure legends

Figure 1. HER2-positive and HER2-negative cases in colorectal cancer

This case was HER2 IHC 3+ in 100% of cancer cells (A, B, C), and was diagnosed as “HER2-positive.” This case was HER2 IHC 3+ in 10% of cancer cells (D, E, F), and was diagnosed as “HER2-negative.”

Hematoxylin and eosin staining, ×1 objective lens (A, D); anti-HER2 staining, ×1 objective lens (B, E), ×20 objective lens (C, F).

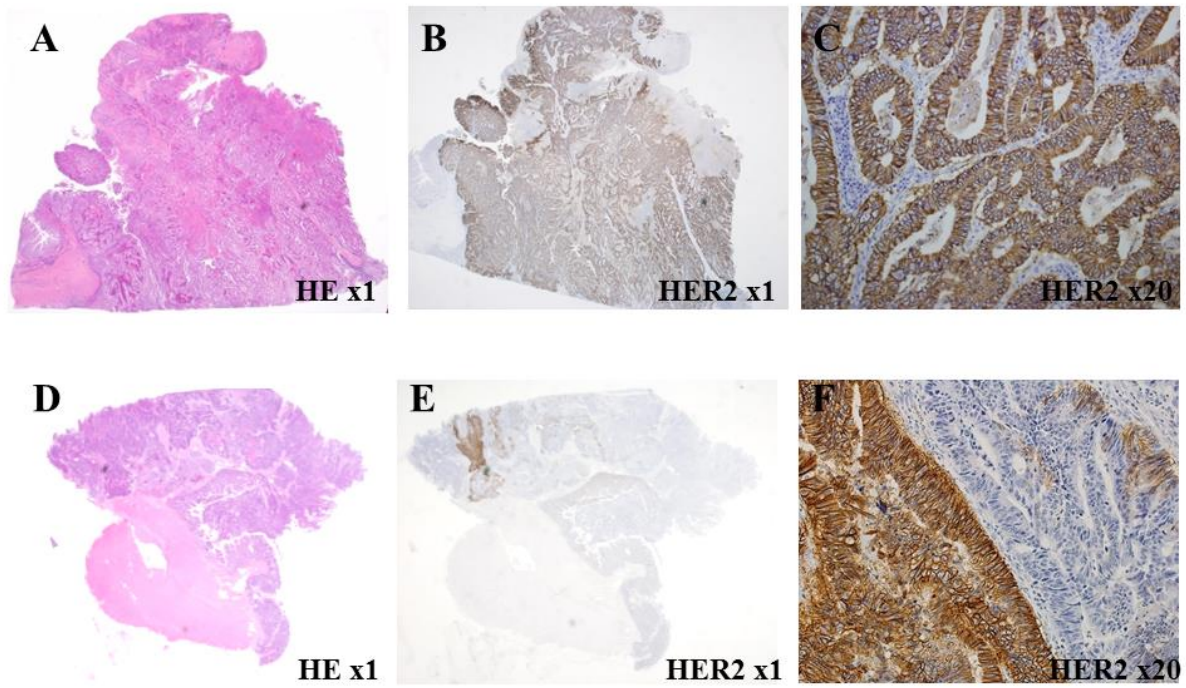
Figure 2. *KRAS* codon 12/13 mutation and *HER2* amplification in 201 patients

There were 9 patients with *KRAS* codon 12/13 wild-type and *HER2* amplification, who were candidates for HER2-targeted therapy.

Figure 3. A case of concordant HER2 status between primary and metastatic sites

This concordant case was diagnosed as IHC 3+ in 100% of cancer cells at both the primary site (A, B) and bone metastasis (C, D).

Hematoxylin and eosin staining, ×20 objective lens (A, C); anti-HER2 staining, ×20 objective lens (B, D).

Fig 1

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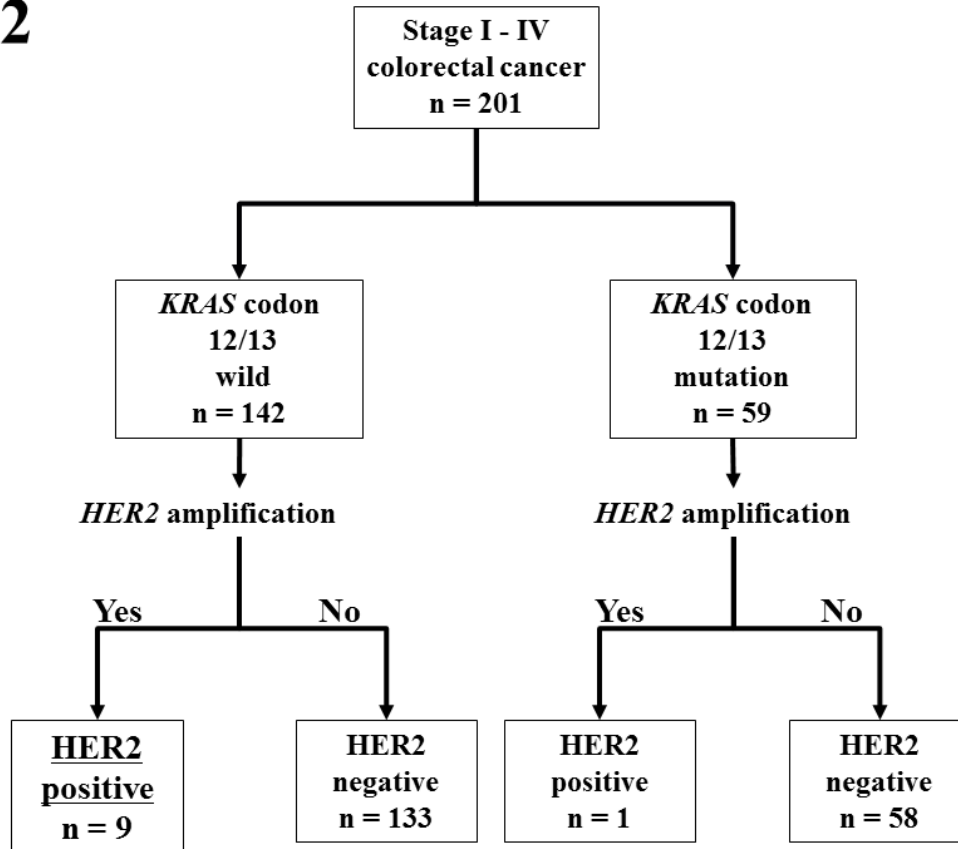
Fig 2

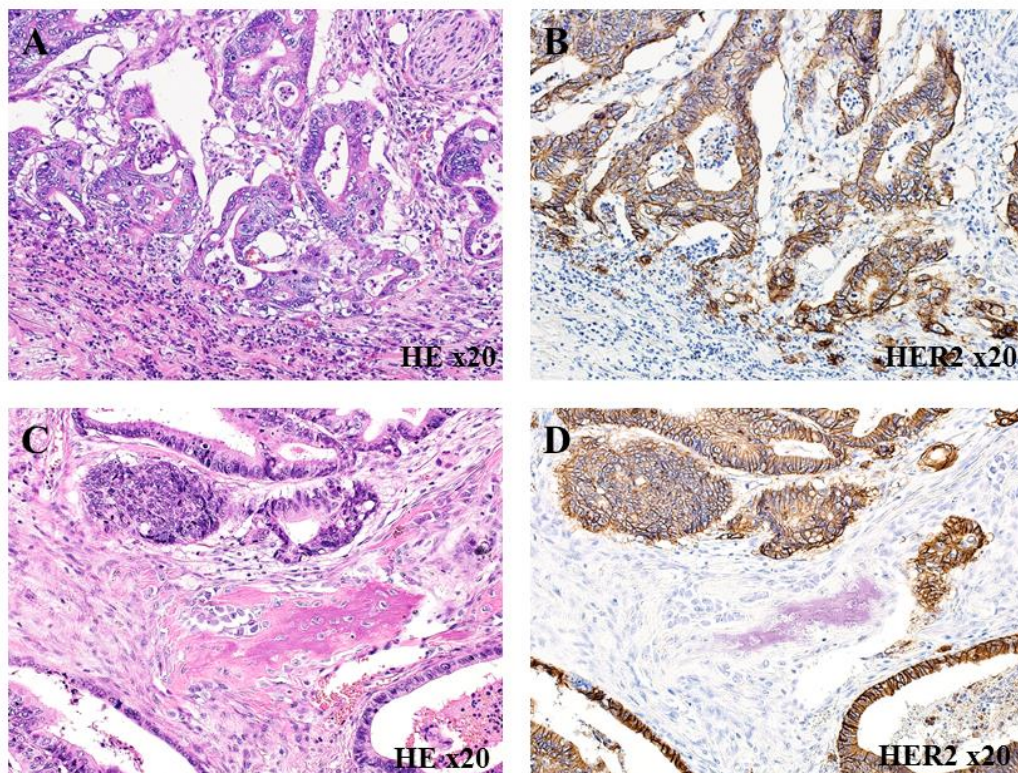
Fig 3

Table 1. Comparison between HER2 status and *HER2* amplification at the primary site

	HER2 status (IHC and FISH)	
	Positive	Negative
<i>HER2</i> amplification (CGS)		
Positive	10	0
Negative	0	191

Abbreviations: HER2, human epidermal growth factor receptor 2; CGS, comprehensive genomic sequencing; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

n = 201, $P < 0.001$.

Table 2. HER2 status and *HER2* amplification/mutation evaluated using CGS

Case No.	Primary site				Metastatic site			
	HER2 status	IHC (% of total area)	FISH	CGS		HER2 status	IHC (% of total area)	FISH
				Amplification	Mutation			
1	Positive	3+ (100%)		Amplified	-			
2	Positive	3+ (100%)		Amplified	-			
3	Positive	3+ (100%)		Amplified	-	Negative	0	
4	Positive	3+ (100%)		Amplified	-			
5	Positive	3+ (100%)		Amplified	-			
6	Positive	3+ (100%)	Positive	Amplified	S310Y			
7	Positive	3+ (60%)		Amplified	-	Positive	3+ (100%)	
8	Positive	3+ (100%)		Amplified	-	Positive	3+ (100%)	
9	Positive	3+ (60%)		Amplified	-			
10	Positive	3+ (80%)		Amplified	-	Positive	3+ (100%)	
11	Negative	2+ (70%)	Negative	-	R678Q			
12	Negative	0		-	V308M			
13	Negative	0		-	A879delinsAE			
14	Negative	2+ (80%)	Negative	-	R647K	Negative	0	
15	Negative	0		-	R678Q			

Abbreviations: HER2, human epidermal growth factor receptor 2; CGS, comprehensive genomic sequencing; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

Table 3. HER2 status and other clinicopathological characteristics

Variable	HER2 status		<i>P</i> -value
	Positive (n = 10)	Negative (n = 191)	
Age (years)			
<65	4	96	0.748
≥65	6	95	
Sex			
Male	7	110	0.526
Female	3	81	
Location			
Right side	1	55	0.289
Left side	9	136	
Tumor size (mm)			
<50	5	83	0.751
≥50	5	108	
T category			
T1, 2	3	21	0.102
T3, 4	7	170	
Histopathological grading			
G1, 2	7	140	0.730
G3	3	51	
Lymphatic invasion			
Absence	6	73	0.195
Presence	4	118	
Venous invasion			
Absence	3	45	0.705
Presence	7	146	
N category			
N0	3	56	0.999
N1, 2	7	135	
M category			
M0	3	87	0.517
M1	7	104	
MLH1/MSH2 status			
Normal	5	86	0.942
Abnormal	1	18	
Medullary type			
Presence	0	5	0.999

Absence	10	186	
Mucinous type			
Presence	0	15	0.999
Absence	10	176	
Crohn-like lymphoid reaction			
Presence	0	31	0.366
Absence	10	160	
Tumor infiltrating lymphocytes ^a			
Presence	0	36	0.214
Absence	10	155	
<i>KRAS</i> codon 12/13			
Wild-type	9	133	0.286
Mutant	1	58	
<i>NRAS</i> codon 12/13			
Wild-type	10	190	0.999
Mutant	0	1	
BRAF V600E			
Wild-type	10	179	0.999
Mutant	0	13	

Abbreviations: HER2, human epidermal growth factor receptor 2.

^a Cut-off value = 10 lymphocytes/5 high power fields.

Table 4. Comparison of HER2 status between 45 primary sites and corresponding metastatic sites

HER2 status at primary site	HER2 status at metastatic site	
	Positive	Negative
Positive	3	1
Negative	0	41

Abbreviations: HER2, human epidermal growth factor receptor 2.
n = 45, $P < 0.001$.

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