Clinicopathologic Characteristics of *HER2*-positive Metastatic Colorectal Cancer and Detection of *HER2* in Plasma Circulating Tumor DNA

Qing Wei,[1,2] Yinjie Zhang,[1] Jing Gao,[1] Jian Li,[1] Jie Li,[1] Yanyan Li,[1] Jun Zhou,[1] Ming Lu,[1] Jifang Gong,[1] Xiaotian Zhang,[1] Lin Shen,[1] Yu Sun,[3] Lianpeng Chang,[4] Xicheng Wang[1]

Abstract

The clinicopathologic characteristics of patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic colorectal cancer were summarized. A targeted next-generation sequencing method was applied to assess HER2 copy number variation and explore the concordance between paired primary tumor samples and plasma circulating tumor DNA (ctDNA). The HER2 concordance rate between tumor samples and ctDNA was 66.7%. Tumor burden changes in ctDNA were consistent with imaging evaluation.

Background: Therapy targeting human epidermal growth factor receptor 2 (*HER2*, also known as *ERBB2*) is an effective approach for *HER2*-positive metastatic colorectal cancer (mCRC). *HER2* status is typically determined using immunohistochemistry and fluorescence in situ hybridization. Circulating tumor DNA (ctDNA) enables noninvasive detection of gene mutations and copy number alterations including *HER2* amplification. **Materials and Methods:** We screened 351 patients with mCRC and studied the clinicopathologic characteristics of *HER2*-positive mCRC. *HER2* expression in tumor samples measured with immunohistochemistry and fluorescence in situ hybridization was compared with *HER2* copy number variation in plasma ctDNA detected by targeted sequence capture covering exons of 170 genes. We also examined the correlation between changes in tumor burden in ctDNA and antitumor response by imaging evaluation during the treatment course. **Results:** Positive *HER2* status was observed in 12 (3.4%) patients (7 males and 5 females), with a median age of 56 years. The *HER2* concordance rate between tumor samples and ctDNA was 66.7% (20/30). Changes in tumor burden in ctDNA during the treatment course correlated with responses on imaging. **Conclusions:** Detection of *HER2* copy number variation in ctDNA may be an alternative option for noninvasive determination of *HER2* status. Tumor burden changes in ctDNA were consistent with imaging evaluation.

Clinical Colorectal Cancer, Vol. 18, No. 3, 175-82
Keywords: Biomarker, CNV, Colorectal adenocarcinoma, ctDNA, Next-generation sequencing

Introduction

HER2, a member of the human epidermal growth factor receptor (HER) family, is a plasma membrane protein with intrinsic tyrosine kinase activity.[1] *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] After dimerization, important signaling pathways such as the mitogen-activated protein kinase and PI3K pathways are activated by *HER2*, resulting in cell duplication and regulation of apoptosis.[3] *HER2* signal transduction can be dysregulated via different mechanisms.

Q.W. and Y.Z. contributed equally to this work as first authors.

[1]Department of Gastrointestinal Oncology, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Peking University Cancer Hospital and Institute, Beijing, China
[2]Department of Medical Oncology, Zhejiang Cancer Hospital, Hangzhou, Zhejiang, China
[3]Department of Pathology, Peking University Cancer Hospital and Institute, Beijing, China
[4]Geneplus-Beijing Institute, Beijing, China

Submitted: Jan 8, 2019; Revised: Apr 17, 2019; Accepted: May 7, 2019; Epub: May 13, 2019

Address for correspondence: Xicheng Wang, PhD, Department of Gastrointestinal Oncology, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Peking University Cancer Hospital and Institute, Fu-Cheng Road 52, Hai-Dian District, Beijing 100142, China
E-mail contact: xicheng_wang@hotmail.com

1533-0028/$ - see frontmatter
https://doi.org/10.1016/j.clcc.2019.05.001

HER2 overexpression, generally resulting from *HER2* gene amplification,[4] is one of the main causes of the constitutive activation of *HER2* signal transduction seen in many cancers.[5] *HER2* overexpression is detected in approximately 20% of breast cancers and 13% to 23% of gastric tumors.[6,7] *HER2* overexpression has also been described in 3% to 5% of colorectal cancers (CRCs).[8,9] Because trastuzumab treatment has been the standard of care for *HER2*-positive (*HER2*$^+$) breast[10] and gastric[11] cancers, there has been great interest in assessing the efficacy of *HER2*-targeted therapy for patients with *HER2*$^+$ CRC.[12] Recently, the *HER2* Amplification for ColorectaL cancer Enhanced Stratification (HERACLES) trial demonstrated efficacy of the combination of trastuzumab and lapatinib in patients with *HER2*$^+$ colorectal carcinoma, and showed that the anti-*HER2* treatment was active in the absence of any chemotherapy backbone in heavily pretreated patients with metastatic disease. The use of trastuzumab for *HER2*$^+$ cases is recognized as a promising treatment strategy for *KRAS* wild-type metastatic CRC.[13] Hence, the *HER2* status of patients with CRC should be evaluated to select candidates for *HER2*-targeted therapy.

The assessment of *HER2* status in breast and gastric cancer usually involves immunohistochemistry (IHC; Hercep Test, Dako) and fluorescence in situ hybridization (FISH; *HER2* FISH pharmDx, Dako) performed on tumor samples. However, some tumors are not accessible for biopsy, and in some cases, obtaining additional biopsy samples is difficult owing to the discomfort suffered by the patient, inherent clinical risks to the patient, or potential surgical complications. Circulating tumor DNA (ctDNA) shed from primary and metastatic cancers may allow non-invasive analysis of the evolution of tumor genomes before and after treatment. The potential role of ctDNA as a novel real-time approach to assess *HER2* status and evaluate treatment response has been studied in breast cancer and gastric cancer.[14,15]

The aim of this study was to elucidate the clinicopathologic characteristics of patients with *HER2*$^+$ metastatic CRC, and to detect *HER2* copy number variation (CNV) of patients with different *HER2* expression levels by IHC using targeted sequence capture (covering exons of 170 genes) to explore the concordance between data for tumor samples and plasma ctDNA. The correlation between changes in tumor burden in ctDNA and anti-tumor response on imaging was also examined.

Materials and Methods

Patient Population

A total of 351 consecutive patients with metastatic colorectal carcinoma in Beijing Cancer Hospital were enrolled from January 2016 to January 2017. All patients gave written informed consent for use of their clinical data and samples in medical research. This study was approved by the Ethics Committee of Peking University Cancer Hospital and performed according to principles of the Declaration of Helsinki.

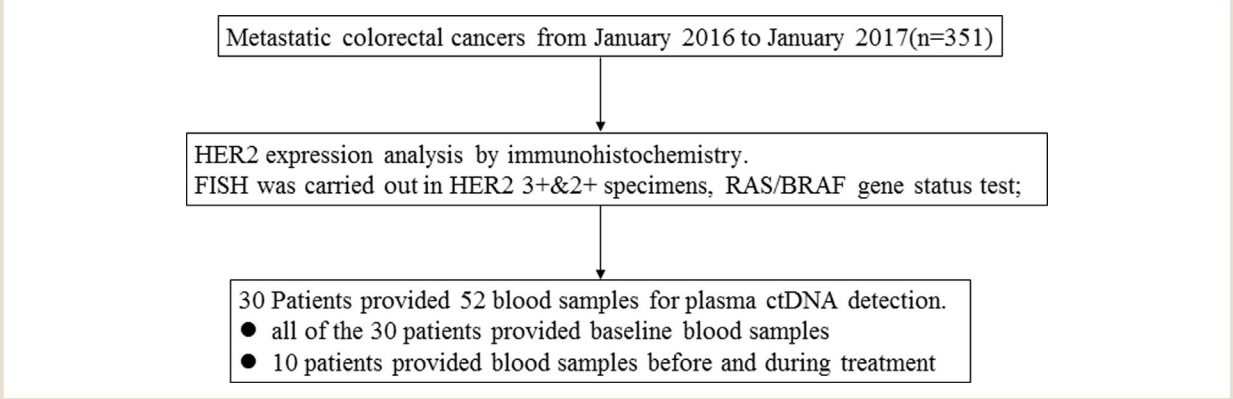
HER2 Status and Clinicopathologic Characteristics of Patients

To analyze the relationship between *HER2* status and clinicopathologic characteristics of patients with CRC, *HER2* expression in all CRC tumor samples was measured by IHC, and extended *RAS* (*KRAS* exons 2, 3 and 4, *NRAS* exons 2, 3 and 4) and *BRAF* (exon 15) genetic testing was performed (Figure 1). Clinical parameters, including age, gender, histologic diagnosis, Eastern Cooperative Oncology Group performance status, and tumor anatomic location at initial presentation were obtained by review of the medical records. The tumor sites were classified as right-sided colon (ileocecal junction, cecum, ascending colon, hepatic flexure, and transverse colon), left-sided colon (splenic flexure, descending colon, and sigmoid colon), or rectum. All tumors were staged according to the TNM staging system of the American Joint Committee on Cancer (7th version, 2009).

HER2 IHC and FISH

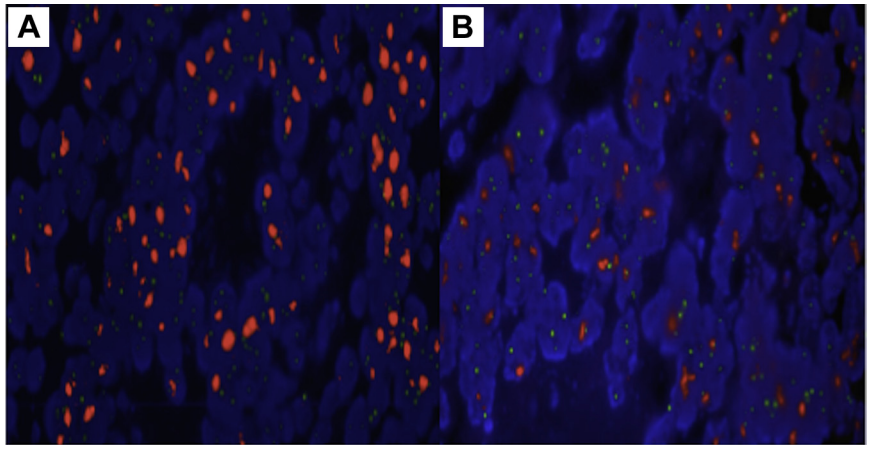
HER2 status of tumor tissue was determined with formalin-fixed and paraffin-embedded (FFPE) samples using routine IHC and FISH methods. According to the American Society of Clinical Oncologists/College of American Pathologists guideline recommendations,[16] IHC scores of 0 and 1+ were considered *HER2*-negative (*HER2*$^-$), whereas an IHC score of 3+ was defined as *HER2*$^+$. An IHC score of 2+ was considered

Figure 1 Flow Chart for Measurement of *HER2* Expression and *RAS/BRAF* Genetic Analysis



Abbreviations: ctDNA = circulating tumor DNA; FISH = fluorescence in situ hybridization; HER2 = human epidermal growth factor receptor 2.

Figure 2 Fluorescence in Situ Hybridization (FISH) of Human Epidermal Growth Factor Receptor 2 (*HER2*) in 2 Patients. Green Signals Represent the Centromeric Region of Chromosome 17, Red Signals Represent the *HER2* Gene on Chromosome 17. A, FISH-positive, FISH:*HER2*/Chr17 = 7.5; B, FISH-positive, FISH:*HER2*/Chr17 = 3.8



equivocal and the sample was subjected to further testing by FISH; a tumor was considered to be *HER2*$^+$ if it had an IHC score of 2+ plus FISH results showing a threshold ratio > 2.0 between the *HER2* CN and chromosome 17 centromere (CEP17). Figure 2 shows *HER2* amplification by FISH in 2 patients with metastatic CRC.

KRAS, NRAS, and BRAF Status

All patients were evaluated for *KRAS*, *NRAS*, and *BRAF* mutations by Sanger sequencing. Genomic DNA was extracted from FFPE sections with ≥ 50% tumor cells (sections with tumor cell content lower than 50% were microdissected) using E.Z.N.A.FFPE DNA Kit (Lot. D3399-01, OMEGA) according to the manufacturer's instructions. All genomic DNA samples were stored at −20°C until further analysis. DNA fragments including the *KRAS*/*NRAS* gene (exon 2/3/4) and exon 15 of the *BRAF* gene were amplified by polymerase chain reaction (PCR). PCR primers and amplified fragments used for *RAS*/*BRAF* testing are detailed in Table 1. Each PCR reaction consisted of 2 μL 10× LA PCR buffer II, 2 μL 2.5 mmol/L dNTPs, 0.1 μL LA Taq (DRR200A, TAKARA), 2 μL genomic DNA, 0.5 μL 10 μmol/L forward primer, and 0.5 μL 10 μmol/L reverse primer in a final volume of 20 μL. The cycling conditions were 95°C for 5 minutes, 45 cycles of 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 20 seconds, and final extension at 72°C for 5 minutes. The detailed sequencing procedures have been reported.[17]

Table 1 PCR Primers and Amplified Fragments Used for *RAS*/*BRAF* Testing

Exon	Primer	Fragments, bp
KRAS		
exon2	F:5'-TACTGGTGGAGTATTTGATAG-3'	248
	R:5'-TGGTCCTGCACCAGTAATATG-3'	
exon3	F:5'-GCACTGTAATAATCCAGACTGTG-3'	222
	R:5'-CCCACCTATAATGGTGAATATCTTC-3'	
exon4	F:5'-ATGACAAAAGTTGTGGACAGGTTTTGA-3'	284
	R:5'-ATGATTTTGCAGAAAACAGATCTGTATTTATTTCAG-3'	
NRAS		
exon2	F:5'-GAACCAAATGGAAGGTCACACT-3'	243
	R:5'-CCTCACCTCTATGGTGGGATC-3'	
exon3	F:5'-TAGCATTGCATTCCCTGTGGTT-3'	258
	R:5'-CCTGTAGAGGTTAATATCCGCAA-3'	
exon4	F:5'-GCCACTGTACCCAGCCTAATCTTG-3'	287
	R:5'-CACATCTCTACCAGAGTTAATCAACTGATGC-3'	
BRAF		
exon15	F:5'-CCTAAACTCTTCATAATGCTTGCTC-3'	211
	R:5'-GTGGAAAAATAGCCTCAATTCTTACC-3'	

Table 2 Correlations Between Clinicopathologic Characteristics of Patients With Colorectal Cancer and *HER2* Status

Characteristic	No. Patients (%)			*P* Value
	All Patients	*HER2* Amplification/ Overexpression	*HER2*-negative	
Total	351	12	339	
Median age, y (range)	57 (26-79)	56 (28-76)	57 (26-79)	.205
Gender				
Male	225	7 (58.3)	218 (64.3)	.906
Female	126	5 (41.7)	121 (35.7)	
Primary tumor				
Right	47	1 (8.3)	84 (24.8)	.077
Left	133	3 (25)	142 (41.9)	
Rectum	171	8 (66.7)	113 (33.3)	
Differentiation				
Moderate-well	303	12 (100)	291 (85.8)	.329
Poor	48	0 (0)	48 (14.2)	
Liver metastases				
Yes	203	10 (83.3)	193 (56.9)	.069
No	148	2 (16.7)	146 (43.1)	
Lung metastasis				
Yes	128	7 (58.3)	121 (35.7)	.195
No	223	5 (41.7)	218 (64.3)	
RAS				
Mutant type	69	1 (8.3)	130 (38.3)	.064
Wild type	282	11 (91.7)	203 (61.7)	
BRAF				
Mutant type	7	0 (0)	7 (2.1)	1
Wild type	344	12 (100)	332 (97.9)	

Abbreviation: HER2 = human epidermal growth factor receptor 2.

Genomic DNA Extraction, Library Preparation, and Sequencing

Genomic DNA was extracted from tumor tissues using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Peripheral blood samples (10 mL) were collected in EDTA Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ), stored at room temperature, and processed within 2 hours. Plasma was separated by centrifugation at 1600 *g* for 10 minutes, transferred to microcentrifuge tubes, and centrifuged at 16,000 *g* for 10 minutes to remove remaining cell debris. A mean volume of 1.5 mL (range, 0.8-2 mL) plasma was obtained after 2 steps of centrifugation. Cell-free DNA (cfDNA) was extracted from tumor tissues using a QIAamp Circulating Nucleic Acid Kit (Qiagen). Peripheral blood lymphocyte genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and sequenced as the matched normal control sample. DNA concentration was measured using a Qubit fluorometer (Invitrogen, Carlsbad, CA) and Bioanalyzer 2100 (Agilent Technologies). The mean concentration of cfDNA in plasma was 52.35 ng/mL.

Peripheral blood lymphocyte gDNA was sheared to 300-bp fragments with a Covaris S2 ultrasonicator, followed by Indexed Illumina NGS library preparation (Illumina, San Diego, CA). cfDNA libraries were prepared using the KAPA DNA Library Preparation Kit (KapaBiosystems, Wilmington, MA). Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Roche NimbleGen, Madison, WI) covering the genes and exons that are most frequently mutated in CRC tumors. DNA sequencing was performed using the HiSeq 3000 Sequencing System (Illumina).

Analysis of ctDNA Abundance and HER2 CNV of ctDNA

Quality control was performed by filtering out the adapter sequences and low-quality reads from the raw data. BWA (version 0.7.12-r1039) was used to align the clean reads to the reference human genome (hg19). Picard (version 1.98) was used to mark PCR duplicates. Realignment and recalibration was performed using GATK (version 3.4-46-gbc02625). Single nucleotide variants were called using MuTect (version 1.1.4) and NChot, software developed in-house to review hotspot variants. Small insertions and deletions were called by GATK. Based on detected single nucleotide variants and small insertions and deletions, PyClone was used to identify clonal mutation, with the highest predicted cancer cell fraction.[18] The mean variable allele frequency of clonal mutations was used to indicate the abundance for a single ctDNA sample. Relative ctDNA abundance at the first therapy evaluation was computed by comparison with abundance

Table 3 Clinicopathologic Characteristics of 30 Patients Who Underwent ctDNA Tests

Characteristic	No. Patients (%)
Total	30
Median age, y (range)	57 (28-77)
Gender	
Male	18 (60)
Female	12 (40)
Primary tumor	
Right	4 (13.3)
Left	10 (33.3)
Rectum	16 (53.3)
Differentiation	
Moderate-well	28 (93.3)
Poor	2 (6.7)
Liver metastases	
Yes	23 (76.7)
No	7 (23.3)
Lung metastasis	
Yes	11 (36.7)
No	19 (63.3)
RAS	
Mutant type	3 (10)
Wild type	27 (90)
BRAF	
Mutant type	0 (0)
Wild type	30 (100)

Abbreviation: ctDNA = circulating tumor DNA.

at pretreatment. Somatic copy number alterations were identified with CONTRA (v2.0.8). In the CONTRA algorithm, base-level log-ratios between the case (ctDNA) and control (gDNA) were taken into consideration to eliminate GC bias and an imbalanced library size effect. Target regions of < 10 bp with depth of coverage < 10 were discarded. For the remaining regions, the mean log ratio was calculated, and the significance *P* value was assigned. Next, circular binary segmentation was applied to the log ratios of regions using default settings. Segmentation results were used for the subsequent analysis. CNV of ctDNA was adjusted with the depth of control gDNA. Normal copy number was expressed to be 2. *HER2* copy number gain was identified using a threshold of 2.3 copies.[19]

Statistics

Statistical analyses were performed with SPSS 19.0 software. The χ^2 or Fisher exact test was used when comparing frequencies between groups. Differences between means of groups were compared by independent sample *t* test. Progression-free survival was defined as the time from treatment to disease progression according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, or death from any cause. Overall survival was defined as the time from diagnosis of metastatic disease to death from any cause. The log-rank test was used to compare Kaplan-Meier survival curves. Non-parametric tests were used for subgroup comparisons (Wilcoxon rank-sum test or Kruskal-Wallis test) and for comparisons between paired samples in each subgroup (Wilcoxon signed-rank test). All tests were 2-sided, and a *P* value less than .05 was considered statistically significant.

Results

Clinicopathologic Characteristics of Patients with HER2$^+$ CRC

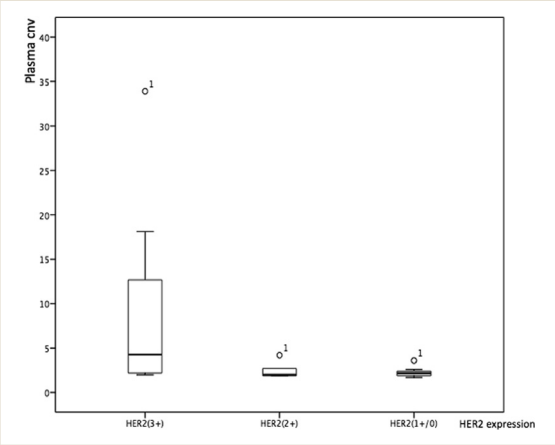
The correlations between clinicopathologic characteristics of CRC patients and *HER2* status are summarized in Table 2. *HER2* amplification/overexpression was identified in 3.4% of cases (12/351; age, 28-76 years; median age, 56 years), including 7 (58.3%) male and 5 (41.7%) female patients. Regarding tumor location, 3 (25.0%) tumors were located on the left-sided colon, 1 (8.3%) tumor was located on the right-sided colon, and 8 (66.7%) tumors were located on the rectum. With regard to the pattern of

Table 4 *HER2* CNV Assessed by IHC/FISH and ctDNA

No.	IHC	FISH[a]	Baseline ctDNA *HER2* CNV[b]	No.	IHC	FISH	Baseline ctDNA *HER2* CNV	No.	IHC	FISH	Baseline ctDNA *HER2* CNV
1	3+	10.32	33.9	12	2+	2.1	4.2	21	0	–	3.6
2	3+	16.7	18.12	13	2+	1.31	2.7	22	0	1.13	2.6
3	3+	14.42	14.42	14	2+	–	2.7	23	0	–	2.4
4	3+	7.25	10.9	15	2+	1	2.26	24	0	–	2.3
5	3+	7.17	5.74	16	2+	1.4	2.02	25	0	–	2.26
6	3+	–	4.26	17	2+	1.09	2	26	0	–	2.1
7	3+	2.31	2.32	18	2+	1.11	1.9	27	0	–	2.1
8	3+	5.1	2.2	19	2+	1.1	1.9	28	1+	0.96	1.9
9	3+	3.8	2.2	20	2+	–	1.9	29	0	–	1.72
10	3+	7.5	2					30	0	0.95	1.7
11	3+	5.27	2								

Abbreviations: CNV = copy number variation; ctDNA = circulating tumor DNA; FISH = fluorescence in situ hybridization; HER2 = human epidermal growth factor receptor 2; IHC = immunohistochemistry.
[a]The unit for FISH is HER2/CEP17 ratio. Tissue samples for IHC and FISH testing were obtained by biopsies or by operative resections at the diagnosis of the malignant disease.
[b]The unit for ctDNA HER2 CNV is estimated HER2 copy-number. All the baseline plasma for ctDNA testing was obtained after the diagnosis of metastatic disease before the initiation of systemic chemotherapy/targeted therapy.

Figure 3 Comparisons of Plasma *HER2* CNV According to Score by Immunohistochemistry. Plasma *HER2* CNV for Patients With Colorectal Cancer With *HER2*(3+) Tumors, *HER2*(2+) Tumors, and Those With *HER2*(1+/0) Tumors Exhibited Different Distribution, But This Was Not Statistically Significant ($P = .057$; Kruskal-Wallis Test)



Abbreviations: CNV = copy number variation; HER2 = human epidermal growth factor receptor 2.

metastasis, 7 (58.3%) cases showed lung involvement, and 10 (83.3%) cases had liver metastases. *RAS* mutation was detected in 1 (8.3%) case, and none of the patients exhibited the *BRAF* V600E mutation.

HER2 Status in Tumor Sample and Serum ctDNA

HER2 staining (3+) by IHC or *HER2* amplification by FISH was considered $HER2^+$ status. *HER2* staining by IHC was scored 3+ in 11 (3.1%) CRC samples, 2+ in 9 (2.6%), and +1/0 in 331 (94.2%). All 9 samples with HER2 staining (2+) by IHC were subjected to *HER2* FISH analysis, with 1 of 9 cases showing *HER2* amplification. Table 3 shows the clinicopathologic characteristics of the 30 patients who provided baseline blood samples for ctDNA testing. Table 4 shows *HER2* CNV assessed by IHC/FISH and ctDNA. Comparisons of plasma *HER2* CNV for patients with *HER2* (3+), (2+), (1+/0) are shown in Figure 3 ($P = .057$; Kruskal-Wallis test). Plasma *HER2* CNV were significantly higher in $HER2^+$ patients than in $HER2^-$ patients ($P = .006$; Wilcoxon rank-sum test).

Table 5 *HER2* Status in Plasma ctDNA With Targeted Capture Sequencing Technique Compared With IHC/FISH of Tumor Section

HER2 in Plasma ctDNA	*HER2* With IHC/FISH in FFPE	
	Positive	Negative
Positive	8	6
Negative	4	12

Abbreviations: ctDNA = circulating tumor DNA; FFPE = formalin-fixed paraffin-embedded; FISH = fluorescence in situ hybridization; HER2 = human epidermal growth factor receptor 2; IHC = immunohistochemistry.

Overall, 66.7% (8 of 12) of patients with *HER2*-amplified CRC were classified as $HER2^+$ in ctDNA, and 66.7% (12 of 18) of patients with *HER2*-nonamplified CRC were classified as *HER2*-negative in ctDNA. The concordance rate of tumor and ctDNA samples was 66.7%. The sensitivity and specificity were 66.7% (8/12) and 66.7% (12/18), respectively (Table 5).

Changes of CNV in ctDNA and Tumor Response Assessed by Imaging Examination

Among 30 patients, 10 patients had samples drawn before and 2 months after first-line systemic treatments (Table 6). In most cases, changes in ctDNA levels during chemotherapy were associated with treatment responses. The pulmonary metastasis lesion of patient no. 4 has shrunk significantly after 2 cycles of trastuzumab plus capacitabine treatment. Nevertheless, patients No. 6 and No. 8 displayed contradictory changes in ctDNA compared with image-based evaluation. For patient 6, the level of ctDNA increased, whereas the imaging showed partial response by RECIST criteria. For patient 8, the level of ctDNA decreased whereas the imaging showed progressive disease by RECIST 1.1 criteria. We did not observe any significant changes in ctDNA *HER2* CNV during treatment. After the exclusion of 3 cases with stable disease or progressive disease, plasma *HER2* CNV before and after treatment was compared for 7 patients who achieved partial response ($P = .237$, Wilcoxon signed-rank test).

Discussion

Several studies have described *HER2* amplification status in patients with CRC. Ingold et al found that 1.6% of 1645 primary colorectal carcinomas were *HER2*/neu-positive.[20] In other studies, *HER2* amplification was seen in 2.2% to 2.7% of CRC.[8,21] We observed *HER2* amplification in 3.7% (20/544) of rectal cancer cases in Chinese patients.[22] In the current study, the $HER2^+$ rate of metastatic colorectal cancer was 3.4% (12/351), which is consistent with earlier studies.[8,21,22]

Seo et al[23] reported that *HER2* gene amplification was more frequently observed in CRCs located in the rectum than in those in the right and left colon. In addition, the HERACLES trial reported that most of the treated patients (23/27; 85%) with *KRAS* codon 12/13 wild-type metastatic CRC had tumors in their distal colon or rectum. In our current study, the majority of the $HER2^+$ tumors were located in the rectum and left-sided colon. This finding might provide insight into enriching the $HER2^+$ CRC population for targeted therapy.

The relationship between *HER2* amplification and *RAS* and *BRAF* status in patients with mCRC has previously been investigated.[24] Two distinct cohorts were tested in this study. Cohort 1 (N = 97) was tested by IHC and dual in situ hybridization (HERamp: *HER2*/CEP17 $\geq$ 2.2), and 14% of patients with *RAS*/*BRAF* wild-type mCRC had *HER2* amplification. These findings were validated in cohort 2 (N = 99), which contained 37 cases of *HER2*-amplified metastatic CRC identified by next-generation sequencing. Richman et al[8] assessed the relationship between *HER2*-amplification/overexpression and *KRAS*/*BRAF*. Pathologic material was obtained from 1914 patients in the QUick And Simple And Reliable (QUASAR) stage II to III trial and 1342 patients in stage IV trials (Fluoxetine or Control Under Supervision [FOCUS] and the Panitumumab, Irinotecan and Ciclosporin in COLOrectal

Table 6 Tumor Response Assessment at First Imaging Evaluation

No.	IHC	*RAS*/*BRAF*	Regimen	First Radiologic Evaluation	ctDNA Amount		ctDNA *HER2* CNV	
					Baseline	First Treatment Evaluation	Baseline	First Treatment Evaluation
1	3+	WT	Chemo + cetuximab	PR	100	1.2	33.9	2.1
2	3+	WT	Chemo	SD	100	138.51	14.42	10.74
3	3+	WT	Chemo	PR	100	1.39	5.74	2.368
4	3+	WT	Chemo + trastuzumab	PR	100	0	2	2.16
5	2+	WT	Chemo	PR	100	5	4.2	2.9
6	2+	WT	Chemo	PR	100	101.6	2.7	2.016
7	2+	WT	Chemo	PR	100	4	2.26	2.5
8	2+	WT	Chemo + cetuximab	PD	100	88.31	2.016	2
9	0	WT	Chemo	SD	100	2	2.3	2.352
10	1+	WT	Chemo + cetuximab	PR	100	7	1.9	2.6

Abbreviations: Chemo = chemotherapy; CNV = copy number variation; ctDNA = circulating tumor DNA; HER2 = human epidermal growth factor receptor 2; IHC = immunohistochemistry; PD = progressive disease; PR = partial response; SD = stable disease; WT = wild type.

cancer therapy trial [PICCOLO]). *HER2* overexpression was associated with *KRAS*/*BRAF* wild-type (WT) status at all stages: in 5.2% WT versus 1.0% mutated tumors ($P < .0001$) for stage IV and 2.1% WT versus 0.2% mutated for stage II to III tumors ($P = .01$). We also showed that most of the *HER2*$^+$ tumors were *RAS* WT (11/12; 91.7%) and that *HER2*$^+$ CRCs were frequently found in the rectum or left-sided colon. Our observations are in line with results of the MyPathway study.[25]

We also detected *HER2* amplification in plasma ctDNA from patients with CRC. Moreover, in most cases, the changes in tumor burden in ctDNA were consistent with imaging evaluation. These results suggested that ctDNA could potentially provide valuable information to characterize *HER2* status and evaluate treatment response in real time.

In our study, the concordance rate of plasma *HER2* CNV by ctDNA and the *HER2* status of tumor samples by IHC/FISH was 66.7%. Reported concordance rates of ctDNA with FFPE for *HER2* amplification vary among different tumor types and studies. Shoda et al[26] showed that the plasma *HER2* ratio correlated with the tumor *HER2* status ($P < .001$) in gastric cancer, with sensitivity and specificity of 0.733 and 0.933, respectively. Kinugasa et al[27] also evaluated *HER2* amplification in FFPE samples and cell-free serum ctDNA in 25 patients with gastric cancer, but the concordance rate of FFPE with ctDNA was not high (62.5%). Gao et al[28] reported that the overall concordance rate was 91.43%, with a sensitivity of 88.89% and specificity of 92.31%. Gevensleben et al[15] reported that the positive and negative predictive value in metastatic breast cancer was 70% and 92%, respectively. These data, together with the concordance rate of 66.7% in our study, imply that the ctDNA technique could be a useful approach to detect *HER2*$^+$ CRC among patients without available tumor samples. Monitoring the dynamics of plasma *HER2* CNV status during the treatment course revealed that plasma *HER2* amplification was not correlated with the therapeutic effects in patients in this study. However, a previous study in Japan demonstrated that the plasma *HER2* status is a sensitive marker that reflects responses and resistance to therapies.[26] This discrepancy may be owing to our small patient sample size, and further studies using a larger number of patients are warranted.

The clinical utility of ctDNA as a cancer biomarker is increasingly being evaluated. Tie et al[29] demonstrated that changes in ctDNA during first-line chemotherapy predict the later radiologic response in patients with metastatic CRC. Patient-specific candidate tissue mutations were detectable in cell-free DNA from the plasma of 48 of 52 patients (concordance, 92.3%). Significant reductions in ctDNA levels (median 5.7-fold; $P < .001$) were observed before cycle 2, which correlated with computed tomography responses at 8 to 10 weeks (odds ratio, 5.25 with a 10-fold ctDNA reduction; $P = .016$). Consequently, analysis of circulating DNA for cancer-specific mutations might prove to be a valuable tool for monitoring tumor progression and responses to therapy and might influence treatment decisions that ultimately improve patient survival. In our study, the changes in ctDNA levels were associated with tumor responses by imaging evaluations with an 80% accuracy rate. Hence, ctDNA measurement has significant potential to complement the standard RECIST-based imaging assessment.

The present study had several limitations. The small patient population and the retrospective nature of the study do not allow us to draw definitive conclusions regarding the effectiveness of detection of *HER2* CNV status in patients with CRC by targeted next-generation sequencing of ctDNA. Another limitation was some inconsistency in *HER2* status between tumor tissue samples and plasma *HER2* CNV. This inconsistency might arise because tumor samples were retrieved after several years, whereas plasma DNA was obtained immediately before treatment. The time interval and administration of adjuvant therapy might affect the genetic profiles of recurrent tumors. In this regard, detection of plasma *HER2* CNV in a real-time manner provides an opportunity to reconsider treatment strategies according to the genetic makeup of ctDNA at different times during the clinical course. In addition, the majority of discrepancies between FISH results and next generation sequencing ctDNA *HER2* CNV are associated with very low tumor cell fraction and limitations in our CNV detection algorithm. Patients with high tumor cell fraction show good correlations between FISH results and *HER2* ctDNA CNV data, whereas those with very low tumor cell fraction show poor correlations between FISH results

and *HER2* ctDNA CNV data. The limit of next generation sequencing approach for cfDNA detection is 0.1%.[30] Low level of cfDNA remains an important challenge for the application of this method. There should be some technical improvements, like including more SNP locations in the panel, to increase sensitivity of CNV detection.

We summarized the clinicopathologic characteristics of patients with *HER2*$^{+}$ metastatic CRC and applied a targeted next generation sequencing method to assess *HER2* CNV and explore the concordance between paired primary tumor samples and plasma ctDNA. In addition, changes in tumor burden in ctDNA were consistent with imaging evaluation, indicating that ctDNA could be used to evaluate the treatment response in real time. Based on our results, ctDNA for *HER2* analysis has potential applications in clinical practice to guide precision medicine.

Clinical Practice Points

- Previous studies about HER2+ colorectal cancer were few, and most of them focused on early stage lesions. We know little about the HER2 status in patients with mCRC.
- The present study explores the clinicopathologic characteristics of HER2+ mCRC in a Chinese population for the first time.
- Overall, the HER2+ rate was 3.4% in 351 patients with mCRC, and the concordance rate of plasma HER2 CNV by ctDNA and the HER2 status of tumor samples by IHC/FISH was 66.7%, indicating that detection of HER2 CNV in ctDNA may be an alternative option for non-invasive determination of HER2 status.
- In addition, the changes in ctDNA levels were associated with tumor responses by imaging evaluations with an 80% accuracy rate in our study. Consequently, analysis of ctDNA for cancer-specific mutations might prove to be a valuable tool for monitoring tumor progression.
- These novel findings will help oncologists optimize personalized treatment and will encourage them to explore new biomarkers for patients with mCRC, which might influence treatment decisions that ultimately improve patient survival.

Acknowledgments

The authors are grateful to the staff at the technical assistance at Geneplus-Beijing Institute. Research registration number: UIN: researchregistry4360, registered at www.researchregistry.com.

Disclosure

The authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clcc.2019.05.001.

References

1. Popescu NC, King CR, Kraus MH. Localization of the human erbB-2 gene on normal and rearranged chromosomes 17 to bands q12-21.32. *Genomics* 1989; 4: 362-6.
2. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *Embo J* 1997; 16:1647-55.
3. Gajria D, Chandarlapaty S. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. *Expert Rev Anticancer Ther* 2011; 11:263-75.
4. Tapia C, Glatz K, Novotny H, et al. Close association between HER-2 amplification and overexpression in human tumors of non-breast origin. *Mod Pathol* 2007; 20:192-8.
5. Scholl S, Beuzeboc P, Pouillart P. Targeting HER2 in other tumor types. *Ann Oncol* 2001; 12(Suppl 1):S81-7.
6. Hanna WM, Ruschoff J, Bilous M, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. *Mod Pathol* 2014; 27:4-18.
7. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* 2008; 19:1523-9.
8. Richman SD, Southward K, Chambers P, et al. HER2 overexpression and amplification as a potential therapeutic target in colorectal cancer: analysis of 3256 patients enrolled in the QUASAR, FOCUS and PICCOLO colorectal cancer trials. *J Pathol* 2016; 238:562-70.
9. Valtorta E, Martino C, Sartore-Bianchi A, et al. Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. *Mod Pathol* 2015; 28: 1481-91.
10. Leonard DS, Hill AD, Kelly L, Dijkstra B, McDermott E, O'Higgins NJ. Anti-human epidermal growth factor receptor 2 monoclonal antibody therapy for breast cancer. *Br J Surg* 2002; 89:262-71.
11. Bang Y-J, Van Cutsem E, Feyereislova A, et al, ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; 376:687-97.
12. Bertotti A, Migliardi G, Galimi F, et al. A molecularly annotated platform of patient-derived xenografts ("xenopatients") identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discov* 2011; 1:508-23.
13. Sartore-Bianchi A, Trusolino L, Martino C, et al. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. *Lancet Oncol* 2016; 17:738-46.
14. Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med* 2013; 368:1199-209.
15. Gevensleben H, Garcia-Murillas I, Graeser MK, et al. Noninvasive detection of HER2 amplification with plasma DNA digital PCR. *Clin Cancer Res* 2013; 19: 3276-84.
16. Hofmann M, Stoss O, Shi D, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008; 52:797-805.
17. Wei Q, Wang X, Gao J, et al. Clinicopathologic and molecular features of colorectal adenocarcinoma with signet-ring cell component. *PLoS One* 2016; 11: e0156659.
18. Murtaza M, Dawson SJ, Pogrebniak K, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat Commun* 2015; 6:8760.
19. Nong J, Gong Y, Guan Y, et al. Circulating tumor DNA analysis depicts subclonal architecture and genomic evolution of small cell lung cancer. *Nat Commun* 2018; 9:3114.
20. Ingold Heppner B, Behrens HM, Balschun K, et al. HER2/neu testing in primary colorectal carcinoma. *Br J Cancer* 2014; 111:1977-84.
21. Marx AH, Burandt EC, Choschzick M, et al. Heterogenous high-level HER-2 amplification in a small subset of colorectal cancers. *Hum Pathol* 2010; 41:1577-85.
22. Wang X, Sun Y, Gao J, Zheng J, Shen L. [Human epidermal growth factor receptor 2 expression in rectal cancer and its clinical implication]. *Zhonghua Wei Chang Wai Ke Za Zhi* 2015; 18:597-601.
23. Seo AN, Kwak Y, Kim DW, et al. HER2 status in colorectal cancer: its clinical significance and the relationship between HER2 gene amplification and expression. *PLoS One* 2014; 9:e98528.
24. Raghav KPS, Overman MJ, Yu R, et al. HER2 amplification as a negative predictive biomarker for anti-epidermal growth factor receptor antibody therapy in metastatic colorectal cancer. *J Clin Oncol* 2016; 34(15 Suppl):3517.
25. Hurwitz H, Raghav KPS, Burris HA, et al. Pertuzumab + trastuzumab for HER2-amplified/overexpressed metastatic colorectal cancer (mCRC): interim data from MyPathway. *J Clin Oncol* 2017; 35(4 Suppl):676.
26. Shoda K, Ichikawa D, Fujita Y, et al. Monitoring the HER2 copy number status in circulating tumor DNA by droplet digital PCR in patients with gastric cancer. *Gastric Cancer* 2017; 20:126-35.
27. Kinugasa H, Nouso K, Tanaka T, et al. Droplet digital PCR measurement of HER2 in patients with gastric cancer. *Br J Cancer* 2015; 112:1652-5.
28. Gao J, Wang H, Zang W, et al. Circulating tumor DNA functions as an alternative for tissue to overcome tumor heterogeneity in advanced gastric cancer. *Cancer Sci* 2017; 108:1881-7.
29. Tie J, Kinde I, Wang Y, et al. Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. *Ann Oncol* 2015; 26:1715-22.
30. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. *N Engl J Med* 2018; 379:1754-65.