HER2 amplification detected in the circulating DNA of patients with gastric cancer: a retrospective pilot study

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Abstract

Background We used real-time quantitative polymerase chain reaction (rqPCR) to detect human epidermal growth factor receptor 2 (*HER2*) amplification in the circulating cell-free DNA (cfDNA) of patients with gastric cancer (GC), which shows the spatial and temporal intrinsic heterogeneity of *HER2* expression/copy number during progression, for liquid biopsy and treatment monitoring.

Methods We first enrolled 52 patients with advanced GC who underwent surgery and 40 healthy volunteers. For patients with GC, plasma cfDNA was obtained before surgery (43 patients) and during postoperative treatment (nine of 43 patients). After ribonuclease P RNA component H1 (*RPPH1*) had been selected as a reference gene for *HER2* CN assessment by rqPCR in GC tumours and plasma, plasma *HER2*-to-*RPPH1* ratios were determined retrospectively in a development cohort and an additional independent validation cohort.

Results The *HER2*-to-*RPPH1* ratio of GC tissues determined by rqPCR was concordant with routinely determined *HER2* status. The plasma *HER2*-to-*RPPH1* ratio was significantly higher for patients with *HER2*-positive tumours than for those with *HER2*-negative tumours. The sensitivity and specificity of the plasma *HER2*-to-*RPPH1* ratio test were 0.539 and 0.967, respectively, in the development cohort, and 0.667 and 1.000, respectively, in the validation cohort. *HER2* amplifications acquired and lost during tumour progression and treatment, respectively, were apparently detected by repeated assessments of plasma *HER2*-to-*RPPH1* ratios during postoperative treatment.

Conclusion Our preliminary data demonstrated the potential clinical use of circulating cfDNA to detect *HER2* amplification as a therapeutic marker to detect and monitor *HER2* CN status for effective molecular targeted therapy in patients with GC.

Keywords *HER2* gene amplification · Gastric cancer · Cell-free DNA · Trastuzumab · Liquid biopsy

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Introduction

Gastric cancer (GC) is one of the most commonly diagnosed cancers and is the sixth-leading cause of cancer-related deaths worldwide [1]. Although surgery remains the mainstay treatment for GC, GC patients often present with advanced, inoperable disease for which conventional chemotherapy reagents have limited success [2]. Thus, there is a need for more effective treatment options.

Human epidermal growth factor receptor 2 (*HER2*) is a transmembrane receptor tyrosine kinase. Its oncogenic activation (e.g. amplification) results in its protein overexpression and subsequent abnormal cell signalling, which

contributes to cancer progression. *HER2* overexpression/amplification was reported in 7–34 % of patients with GC, and in a meta-analysis it was associated with more aggressive disease and poorer survival [3, 4]. A recent phase III trial of trastuzumab for patients with *HER2*-positive advanced GC (ToGA trial) showed that the addition of trastuzumab to chemotherapy produced a statistically significant benefit in terms of overall survival versus chemotherapy alone [3]. On the basis of these results, the European Medicines Agency approved trastuzumab for the treatment of metastatic adenocarcinomas of the stomach in 2010. However, it noted that trastuzumab should be used only for patients with metastatic GC whose tumours overexpress *HER2* as routinely defined by an immunohistochemistry (IHC) score of 3+ or 2+ with a positive fluorescent in situ hybridization (FISH) result. Therefore, an accurate diagnosis of *HER2* status in GC has become increasingly important for deciding on the treatment in the clinical setting.

Two important issues for *HER2*-targeted therapy in GC are (1) inaccurate assessments of *HER2* status owing to intratumoral heterogeneity [5–7] or genetic differentiation accompanied by neoplastic progression, which results in inappropriately choosing patients for *HER2*-targeted therapy, and (2) therapeutic resistance owing to selection of *HER2*-amplified subclones accompanied by *HER2*-targeted therapy. Indeed, intratumoral *HER2* heterogeneity is more frequent in GC than in breast cancer (BC) [5, 8], and the overall rate of response to *HER2*-targeted therapy in patients with *HER2*-positive GC is 47.3 % [3], indicating that there are a considerable proportion of these patients who may be refractory to *HER2* inhibition, even when the drug target is present.

Analysis of plasma or serum circulating cell-free DNA (cfDNA), one type of ‘liquid biopsy’, has attracted clinicians’ interest as a new approach that is suitable for repeated measurements of *HER2* amplification for the purpose of treatment monitoring and without the need for an invasive biopsy [9–12]. In a study of *HER2*-positive metastatic BC, reduced *HER2* amplification detected in plasma after first-line trastuzumab therapy in combination with chemotherapy was correlated with improved response and overall survival [13]. Although detection of amplification or methylation of cancer-related genes using circulating cfDNA in GC has also been reported [14, 15], no studies have examined the dynamics of plasma *HER2* amplification in cfDNA before and after surgery and during adjuvant chemotherapy with or without trastuzumab in GC [7].

The dynamics of *HER2* amplification detected in circulating cfDNA as determined by real-time quantitative polymerase chain reaction (rqPCR) may be of diagnostic and prognostic significance. In this pilot study, we focused on a type of ‘liquid biopsy’ using repeated plasma cfDNA sampling, and assessed the performance of rqPCR-based *HER2*

copy number (CNs) analysis as a non-invasive approach in advanced GC for the purpose of better molecular prediction of therapeutic effects and treatment monitoring.

Materials and methods

Patients and samples

Our study protocol was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki. All subjects gave their written informed consent prior to their participation. Samples were blinded for analysis and patients understood that the results would not be made available to them. The development cohort (Table 1) and the independent validation cohort to assess the cut-off value for plasma rqPCR-based *HER2* CN analysis (Table S1) included 52 and 25 patients with GC, respectively, who underwent surgery and had IHC *HER2* score determinations between January 2008 and July 2013 at the Kyoto Prefectural University of Medicine Hospital (Fig. 1). Among these patients, preoperative plasma samples were collected from 43 patients (Table 2). For 48 patients, resected cancer specimens were obtained and fixed in buffered formalin and embedded in paraffin for routine pathology examinations (Table S2). Both preoperative plasma samples and tumour specimens were obtained from 39 patients. Control plasma samples were also obtained from 40 adult healthy volunteers by standard antecubital venous puncture.

Patient demographic data and details for tumour recurrence and subsequent management were recorded. Computed tomography (CT) was performed and the findings were reviewed in a blinded manner to document treatment responses according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 [16]. Pathological classification of tumours was determined according to Union for International Cancer Control classifications [17]. Tissue *HER2* status was determined with formalin-fixed and paraffin-embedded (FFPE) samples using routine IHC and FISH methods. Following the American Society of Clinical Oncologists/College of American Pathologists guideline recommendations, an IHC scores of both 0 and 1+ were considered *HER2* negative, whereas an IHC score of 3+ was defined as *HER2* positive. An IHC score of 2+ was considered equivocal; a tumour was considered to be *HER2* positive if it had an IHC score of 2+ plus FISH with a threshold ratio of more than 2.0 between the *HER2* CN and chromosome 17 centromere (CEP17). Trastuzumab therapy was routinely administered to *HER2*-positive patients; however, two *HER2*-positive patients with GC refused this therapy and one *HER2*-negative patient with GC received this therapy (Table 1).

Table 1 Clinicopathological features and their relationship with the tumour human epidermal growth factor receptor 2 (*HER2*) status in 52 patients with gastric cancer from the development cohort

Variables	Number	Tumour <i>HER2</i> status ^a		
		Negative (%)	Positive (%)	<i>P</i> ^b
Total	52	38 (73.1)	14 (26.9)	
Gender				
Male	31	23 (74.2)	8 (25.8)	0.8258
Female	21	15 (71.4)	6 (28.6)	
Age (years) ^c		65.6 ± 12.1	64.9 ± 8.7	0.7027
Histopathologically predominant ^d				
Differentiated	19	12 (63.2)	7 (36.8)	0.2261
Undifferentiated	33	26 (78.8)	7 (21.2)	
Size ^c		96.8 ± 53.1	92.4 ± 41.9	0.8527
T stage ^e				
T2/3	17	12 (70.6)	5 (29.4)	0.7790
T4	35	26 (74.3)	9 (25.7)	
N stage ^e				
N0	11	10 (90.9)	1 (9.1)	0.0001
N1/2	27	24 (88.9)	3 (11.1)	
N3	14	4 (28.6)	10 (71.4)	
Stage ^e				
II	10	9 (90.0)	1 (10.0)	0.3168
III	40	28 (70.0)	12 (30.0)	
IV	2	1 (50.0)	1 (50.0)	
Lymphatic invasion				
0–2	12	10 (83.3)	2 (16.7)	0.3437
3	40	28 (70.0)	12 (30.0)	
Venous invasion				
0–2	40	29 (72.5)	11 (27.5)	0.8633
3	12	9 (75.0)	3 (25.0)	
<i>HER2</i> expression by IHC				
0	16	16 (100.0)	0 (0.0)	<0.0001
1	15	15 (100.0)	0 (0.0)	
2	9	7 (77.8)	2 (22.2)	
3	12	0 (0.0)	12 (100.0)	
Neoadjuvant chemotherapy				
Present	5	2 (40.0)	3 (60.0)	0.1002
Absent	47	36 (76.6)	11 (23.4)	
Trastuzumab therapy ^f				
Present	13	1 (7.7)	12 (92.3)	<0.0001
Absent	39	37 (94.9)	2 (5.1)	

^a Tumour *HER2* status was determined by an immunohistochemistry (IHC) score of 3 or an IHC score of 2 with a positive fluorescent in situ hybridization result as described in the text.

^b *P* values are from the χ^2 test or Fisher's exact test, and were statically significant at less than 0.05. Statistically significant values are given in *italics*.

^c Mean ± standard deviation

^d 'Differentiated' corresponds to tubular adenocarcinoma or papillary adenocarcinoma, and 'undifferentiated' corresponds to poorly differentiated adenocarcinoma, signet-ring carcinoma or mucinous adenocarcinoma.

^e Disease stage was defined in accordance with the Union for International Cancer Control seventh tumour–lymph node–metastasis (TNM) classification using surgical-pathologic findings.

^f Trastuzumab therapy for patients with *HER2*-positive gastric cancer was performed on the basis of the European Medicines Agency criteria.

To assess rqPCR-based methods, genomic DNA from GC cell lines (Table S3), SK-BR-3 BC cells (American Type Culture Collection, Manassas, VA, USA) and normal leucocytes (Promega, Madison, WI, USA) were used.

Preparation and DNA isolation from plasma and tissues samples

A 3-ml EDTA-blood sample was obtained from each patient before surgery and from 40 healthy volunteers.

In nine of 43 patients from the development cohort and 11 of 25 patients from the validation cohort, samples were also collected after surgery and/or during adjuvant therapy. Plasma was immediately separated from the cellular fraction using a three-spin protocol as described elsewhere [18], and was stored at −80 °C until further processing. Cell-free DNA (cfDNA) was isolated from 200 µl of a plasma sample with a QIA-amp MinElute virus spin kit (Qiagen, Hilden, Germany).

Fig. 1 Study design. *cfDNA* cell-free DNA, *CN* copy number, *FFPE* formalin fixed and paraffin embedded, *GC* gastric cancer, *HER2* human epidermal growth factor receptor 2, *rqPCR* real-time quantitative polymerase chain reaction

Genomic DNA of cancerous and adjacent non-cancerous gastric tissues was extracted from three slices (5- μ m thick) of FFPE tissues using a ReliaPrep FFPE gDNA Miniprep system (Promega).

Quantitative analysis of *HER2* CNs using rqPCR

All rqPCR amplifications were performed in triplicate using an Applied Biosystems 7500 real-time PCR system (Life Technologies, Carlsbad, CA, USA). The final PCR mixture (10 μ l) contained 5 μ l of TaqMan universal PCR master mix (Life Technologies), 0.5 μ l of TaqMan CN assay (Life Technologies) for *HER2* (Hs05499477_cn), ribonuclease P RNA component H1 (*RPPH1*) (TaqMan reference assay), *TAOK1* (Hs02294672_cn) or *EFTUD2* (Hs00364067_cn) and 4.5 μ l of a DNA sample.

To determine *HER2* gene amplification (*HER2* to reference gene ratio), the ΔC_t value was determined (average C_t value of the target gene minus the average C_t value of the reference gene) and was used to calculate the $\Delta\Delta C_t$ value for each DNA sample with a mean relative quantitation (RQ) value. The RQ values for GC tissues and

plasma cfDNA from patients with GC were derived from 44 non-tumour gastric tissues [$RQ = 1.00 \pm 0.2953$; mean $\pm$ standard deviation (SD)] and a panel of plasma cfDNA samples from 40 healthy volunteers ($RQ = 1.00 \pm 0.3162$), respectively, used as experimental calibrators. RQ values were calculated from $2^{-\Delta\Delta C_t}$. A Z score for plasma sample analysis was calculated as follows: $Z \text{ score} = (RQ \text{ patient plasma sample} - RQ \text{ average normal plasma panel}) / \text{average SD of normal plasma panel}$.

Identifying a reference region

We obtained CN data as determined by microarray (Affymetrix SNP6.0 array) from the publically available Cancer Cell Line Encyclopedia (CCLE) database (BROAD Institute; <http://www.broadinstitute.org/>) for 20 GC cell lines, which were available for our rqPCR analysis. To assess candidate reference genes for rqPCR-based *HER2* CN testing, we compared our rqPCR results with CN data from the CCLE database in cell lines, and also performed rqPCR for normal gastric tissues and normal control plasma cfDNA.

Table 2 Clinicopathological features of 43 gastric cancer patients with analysis of plasma real-time quantitative polymerase chain reaction (rqPCR) *HER2*-to-*RPPH1* copy number ratio from the development cohort

Variables	Number	Tumour <i>HER2</i> status ^a					Plasma <i>HER2</i> amplification ^b				
		Negative		Positive		<i>P</i> ^c	Absent		Present		<i>P</i> ^c
		<i>n</i>	%	<i>n</i>	%		<i>n</i>	%	<i>n</i>	%	
Total	43	30	69.8	13	30.2		35	81.4	8	18.6	
Gender											
Male	25	17	68.0	8	32.0	0.7656	22	88.0	3	12.0	0.1921
Female	18	13	72.2	5	27.8		13	72.2	5	27.8	
Age (years) ^d		66.4 ± 10.7		65.9 ± 8.1		0.8635	66.5 ± 10.3		65.4 ± 8.3		0.7669
Histopathologically predominant ^e											
Differentiated	16	10	62.5	6	37.5	0.4275	12	75.0	4	25.0	0.4127
Undifferentiated	27	20	74.1	7	25.9		23	85.2	4	14.8	
Size ^d		95.0 ± 51.5		98.0 ± 41.3		0.5620	89.5 ± 49.2		130.0 ± 22.4		0.0236
T stage ^f											
T2/3	13	9	69.2	4	30.8	0.9598	11	84.6	2	15.4	0.7172
T4	30	21	70.0	9	30.0		24	80.0	6	20.0	
N0	7	6	85.7	1	14.3	0.0005	6	85.7	1	14.3	0.0637
N1/2	24	21	87.5	3	12.5		22	91.7	2	8.3	
N3	12	3	25.0	9	75.0		7	58.3	5	41.7	
Stage ^f											
II	6	5	83.3	1	16.6	0.6221	6	100.0	0	0.0	0.1723
III	35	24	68.6	11	31.4		28	80.0	7	20.0	
IV	2	1	50.0	1	50.0		1	50.0	1	50.0	
0–2	10	9	90.0	1	10.0	0.0865	10	100.0	0	0.0	0.0291
3	33	21	63.6	12	36.4		25	75.8	8	24.2	
Venous invasion											
0–2	33	23	69.7	10	30.3	0.9854	26	78.8	7	21.2	0.3993
3	10	7	70.0	3	30.0		9	90.0	1	10.0	
HER2 expression by IHC											
0	11	11	100.0	0	0.0	<0.0001	11	100.0	0	0.0	0.0007
1	13	13	100.0	0	0.0		13	100.0	0	0.0	
2	8	6	75.0	2	25.0		6	75.0	2	25.0	
3	11	0	0.0	11	100.0		5	45.5	6	55.5	
Neoadjuvant chemotherapy											
Present	5	2	40.0	3	60.0	0.1406	3	60.0	2	40.0	0.2302
Absent	38	28	73.7	10	26.3		32	84.2	6	15.8	
Trastuzumab therapy ^g											
Present	13	1	7.7	12	92.3	<0.0001	6	46.2	7	53.8	0.0001
Absent	30	29	96.7	1	3.3		29	96.7	1	3.3	

^a Tumour human epidermal growth factor receptor 2 (*HER2*) status was determined by an immunohistochemistry (IHC) score of 3 or an IHC score of 2 with a positive fluorescent in situ hybridization result as described in the text.

^b Plasma *HER2* amplification was determined by the plasma rqPCR *HER2*-to-*RPPH1* copy number ratio test with 3.05 as a cut-off value for the Z score as described in “Quantitative analysis of *HER2* CNs using rqPCR”.

^c *P* values are from the χ^2 or Fisher’s exact test, and were statically significant at less than 0.05. Statistically significant values are given in italics.

^d Mean ± standard deviation

^e ‘Differentiated’ corresponds to tubular adenocarcinoma or papillary adenocarcinoma, and ‘undifferentiated’ corresponds to poorly differentiated adenocarcinoma, signet-ring carcinoma or mucinous adenocarcinoma.

^f Disease stage was defined in accordance with the Union for International Cancer Control seventh tumour–lymph node–metastases (TNM) classification using surgical-pathologic findings.

^g Trastuzumab therapy for patients with *HER2*-positive gastric cancer was performed on the basis of the European Medicines Agency criteria.

Statistical analysis

Spearman correlation coefficients were determined to assess the associations between CN data determined by different methods. Non-parametric tests were used for subgroup comparisons (Wilcoxon t test or Kruskal–Wallis test) and for comparisons between paired samples in each subgroup (Wilcoxon signed-rank test). The trends for tissue

and plasma *HER2*-to-*RPPH1* ratios across IHC *HER2* scores were assessed using the Jonckheere–Terpstra test. Receiver operating characteristic (ROC) curves and the areas under the ROC curves (AUC) were used to assess the most appropriate cut-off values for detecting *HER2*-positive GC, which was determined using a Youden index [19]. A χ^2 test or Fisher's exact test was used to assess associations between plasma *HER2*-to-*RPPH1* ratio results and

Fig. 2 Correlations between tumour *HER2* status and the rqPCR *HER2*-to-*RPPH1* CN assay results of tissues and plasma samples in patients with GC. **a** Comparisons of tissue rqPCR *HER2*-to-*RPPH1* CN ratios (relative quantitation, RQ) in GC non-tumour tissues, *HER2*-positive tumours and *HER2*-negative tumours. Tumour *HER2* status was determined on formalin-fixed and paraffin-embedded sections by routine immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) methods, and an IHC score of 3+ or an IHC score of 2+ plus FISH with a threshold ratio of more than 2.0 between the *HER2* CN and the chromosome 17 centromere. The average *HER2*-to-*RPPH1* CN ratio of GC non-tumour tissues was set to 1 [standard deviation (SD) 0.2953]. **b** Tissue rqPCR *HER2*-to-*RPPH1* CN ratios assessed in GC tumour samples from 48 patients, including 13 with *HER2*-positive tumours and 35 with *HER2*-negative tumours (*bottom*). A cut-off value of 2.13 was determined using receiver operating characteristic (ROC) analysis with an area under the curve (AUC) of 0.803 (*top*). **c** Comparisons of plasma rqPCR *HER2*-to-*RPPH1* CN ratios (Z scores) for healthy volunteers and patients with GC with *HER2*-positive tumours or *HER2*-negative tumours. Z scores were calculated using the mean and SD of RQ values in a normal plasma panel. Tumour *HER2* status was determined as described for **a**. **d** Plasma rqPCR *HER2*-to-*RPPH1* CN ratios (Z scores) assessed in GC tumour samples from 43 patients, including 13 with *HER2*-positive tumours and 30 with *HER2*-negative tumours (*bottom*). A cut-off value of 3.05 was determined using ROC analysis with an AUC of 0.746 (*top*)

clinicopathological factors. All statistical tests except for paired tests were two sided. Statistical significance was accepted for $P < 0.05$.

Results

Reference gene selection for relative *HER2* CN analysis using rqPCR in GC

Our study design for assessing plasma *HER2* amplification in patients with GC is shown in Fig. 1. We first optimized our rqPCR assay for *HER2* CNs relative to reference gene CNs in GC. We initially chose three candidate genes, *RPPH1* (14q11.2), *TAOK1* (17q11.2) and *EFTUD2* (17q21.31), on the basis of previous reports [11, 20–22] to avoid false increases or decreases in *HER2* CN ratios owing to their normal CN variations as well as frequent CN alterations in GC. In microarray-based CN data obtained from the publically available CCLE database using bioinformatics, the smallest variation was found for *RPPH1* among these three candidates in 20 GC cell lines (2.107 ± 0.4045 , 2.023 ± 0.4694 and 2.207 ± 0.4617 for *RPPH1*, *TAOK1* and *EFTUD2*, respectively; mean $\pm$ SD; Table S3).

In these 20 GC cell lines, the highest correlation between the ratio of *HER2* relative to each reference gene as determined by our rqPCR method and the *HER2* CN data from the CCLE database was for *RPPH1* (*HER2*-to-*RPPH1* ratio) among these three genes (Fig. S1a). The

highest correlation between the ratio of *HER2* CN relative to each reference gene and the ratio of *HER2* relative to average values of these three reference genes determined in 20 GC cell lines by rqPCR was also for *RPPH1* ratios (Fig. S1b). These results suggested that *RPPH1* would be a useful probe as a reference gene for our *HER2* CN analysis in GC. In addition, the stability of the *HER2*-to-*RPPH1* ratio was validated using 44 non-tumour DNA samples from patients with GC and 40 normal plasma cfDNA samples (Fig. S1c). The rqPCR *HER2*-to-*RPPH1* ratios in serial dilutions starting from 10 ng of a standard sample set prepared by mixing two DNAs without and with *HER2* amplification, which were from normal leucocyte DNA and SK-BR-3 cells, respectively, indicated that these results were reproducible up to a 256-fold dilution (Fig. S1d).

HER2 CN analysis of GC tissues by rqPCR

The mean RQ value of the *HER2*-to-*RPPH1* ratios in 44 non-tumour tissues determined by rqPCR was set at 1.00. Then, tumour *HER2* CN was assessed as the *HER2*-to-*RPPH1* ratio in each GC tissue relative to non-tumour tissues. The GC *HER2*-to-*RPPH1* ratios were correlated with IHC *HER2* scores ($P = 0.000798$, Kruskal–Wallis test; $P = 0.0000217$, Jonckheere–Terpstra trend test; Fig. S2a), and were significantly higher in *HER2*-positive tumours than in *HER2*-negative tumours ($P = 0.0015$, Wilcoxon t test; Fig. 2a). The cut-off value for the tumour *HER2*-to-*RPPH1* ratio to detect *HER2* amplification in GC was determined from ROC analysis with an AUC of 0.803 [95 % confidence interval (CI) 0.653–0.956]. The cut-off value, sensitivity and specificity were 2.13, 0.692 and 0.800, respectively (Fig. 2b).

The results obtained with the rqPCR *HER2*-to-*RPPH1* ratio assay for GC were highly concordant with *HER2* status as determined by routine IHC and FISH (concordance of 37/48, 0.771). In addition, the results with the rqPCR *HER2*-to-*RPPH1* ratio assay were highly concordant with the *HER2* to chromosome 17 centromere ratio determined by FISH (Fig. S2b). In eight IHC 2+ tumours, however, two of two FISH-positive tumours (100 %) and two of six FISH-negative tumours (33.3 %) were tissue rqPCR amplification negative; whereas four of six FISH-negative tumours (66.7 %) were tissue rqPCR amplification positive (Table S4, Fig. S2b).

Since samples for these two methods were not obtained from the exact same place, these results suggested high intratumoral heterogeneity of *HER2* CN and expression in GC tumours, especially in those with borderline *HER2* status.

HER2 amplification detection in circulating cfDNA

To more objectively evaluate whether the plasma rqPCR *HER2*-to-*RPPH1* ratio (RQ) for an individual patient's sample deviated from the patterns of normal samples, comparative determinations of plasma *HER2*-to-*RPPH1* ratios in patient with GC were made by setting the mean RQ value of the plasma *HER2*-to-*RPPH1* ratios of 40 healthy volunteers (control samples) to 1.00, after which Z scores were calculated on the basis of the mean and SD of this control sample set [23]. The Z scores of plasma *HER2*-to-*RPPH1* ratios were correlated with IHC *HER2* scores using the Kruskal–Wallis and Jonckheere–Terpstra trend tests ($P = 0.0443$ and $P = 0.00429$, respectively; Fig. S2c), and were significantly higher in patients with *HER2*-positive tumours than in those with *HER2*-negative tumours ($P = 0.0116$; Wilcoxon t test; Fig. 2c). The cut-off value for Z scores of this plasma *HER2*-to-*RPPH1* ratio test to detect *HER2* amplification in GC was determined using ROC analysis with an AUC of 0.746 (95 % CI 0.560–0.932). The cut-off value, sensitivity and specificity were 3.05, 0.539 and 0.967, respectively (Fig. 2d). In 25 patients in the independent validation cohort, including three patients with *HER2*-positive tumours, the sensitivity and specificity were 0.667 and 1.000, respectively, at the cut-off value of 3.05 for Z scores of the plasma *HER2*-to-*RPPH1* ratio test (Table S1).

In 39 GC patients, the Z scores for plasma *HER2*-to-*RPPH1* ratios were correlated with tumour *HER2*-to-*RPPH1* RQ values ($r = 0.424$, 95 % CI 0.125–0.652, $P = 0.00721$). A comparison of the Z scores for the plasma *HER2*-to-*RPPH1* ratios before and after surgery in patients from the development and validation cohorts supported the accuracy and reproducibility of this method (Fig. 3). Decreases in the Z scores for plasma *HER2*-to-*RPPH1* ratios after surgery were observed in patients with plasma *HER2* amplification before surgery, but not for patients without plasma *HER2* amplification in both cohorts, and the decrease observed in *HER2*-positive patients was statistically significant even though in a small number of patients ($P = 0.0122$).

Possible clinical impact of plasma *HER2* testing for GC

The correlations between clinicopathological characteristics of patients with GC and the results of *HER2* status determined by routine methods and plasma and tumour rqPCR *HER2*-to-*RPPH1* ratio assays are summarized in Tables 1, 2 and Table S2. Although samples for plasma and/or tumour rqPCR *HER2*-to-*RPPH1* ratio tests were available for only some of the GC patients, the correlations between patient characteristics and tumour *HER2* status appeared to be similar among all 52 patients on the basis of

48 patients who were analysed for tissue rqPCR *HER2*-to-*RPPH1* ratios and 43 patients who were analysed for plasma rqPCR *HER2*-to-*RPPH1* ratios.

As with routine-method-based tumour *HER2* status, the plasma *HER2* amplification results tended to be associated with N stage ($P = 0.0637$; Table 2). A similar tendency was found for lymphatic invasion ($P = 0.0865$ vs $P = 0.0291$ for routine methods vs plasma rqPCR CN assay, respectively). However, unlike routine-method-based tumour *HER2* status, the plasma *HER2* amplification results were significantly associated with tumour size ($P = 0.0236$). For 13 patients with *HER2*-positive tumours and whose plasma cfDNA samples were available for rqPCR CN testing, the Z scores of plasma *HER2*-to-*RPPH1* ratios were also associated with tumour size ($P = 0.0285$; Table S5). These results suggested that the Z score of the plasma *HER2*-to-*RPPH1* ratio reflected the level of *HER2*-positive cancer cells as well as the degree of *HER2* amplification in tumour cells, at least in part.

Discrepancies among *HER2* amplification status determined by these three tests were found for some patients (Fig. 4a). By focusing on the discrepancies between routine-method-based tumour *HER2* status and plasma rqPCR *HER2*-to-*RPPH1* ratio testing, we made a detailed analysis of subgroups to assess the possible usefulness and limitations of plasma *HER2* CN testing for GC treatment.

For 13 patients who were treated with trastuzumab, 12 were *HER2* positive by routine methods, and seven patients showed plasma *HER2* amplification (Table 3). Interestingly, a more effective response to trastuzumab (partial response) was found in all seven patients with plasma *HER2* amplification, whereas a less effective response to this treatment (stable disease or progressive disease) was found for four of six patients (three stable disease patients and one progressive disease patient) without plasma *HER2* amplification ($P = 0.0210$; Fisher's exact test). We found that rqPCR-based tissue *HER2* amplification was not associated with the therapeutic response.

We also assessed the clinical courses of three patients, MK412, MK418 and MK489, whose long-term follow-up along with repeated measurements of plasma *HER2* CNs was performed after surgery (Fig. 4b–d). Patient MK489, with an *HER2*-positive tumour, had a high Z score for the plasma *HER2*-to-*RPPH1* ratio before surgery, and was treated with trastuzumab and docetaxel for recurrence of abdominal lymph node metastasis that was detected by CT at 3 months after surgery (Fig. 4b). This patient had a continuous decrease in the Z score for the plasma *HER2*-to-*RPPH1* ratio along with a partial response to treatment for metastatic lymph nodes for more than 2 years. Patient MK412, with an *HER2*-negative tumour, had a low Z score for the plasma *HER2*-to-*RPPH1* ratio before surgery (Fig. 4c). The Z score for the plasma *HER2*-to-*RPPH1*

Fig. 3 Comparisons of Z scores for plasma *HER2*-to-*RPPH1* CN ratios before and after surgery. Since sets of plasma cfDNA before and after surgery were available only in a limited number of patients, Z scores for plasma *HER2*-to-*RPPH1* CN ratios were compared before and after surgery for five patients with GC with plasma *HER2* amplification before surgery (*left*) and 15 patients without plasma

HER2 amplification before surgery (*right*) from the development and validation cohorts. A similar pattern was observed in both cohorts. Black circles and grey circles represent values for each patient from the development and validation cohorts, respectively, at each time point

ratio increased gradually and a GC relapse occurred as multiple liver metastases during follow-up. These metastatic tumours showed no response to irinotecan plus cisplatin therapy. Patient MK418, with an *HER2*-positive tumour, had a low Z score for the plasma *HER2*-to-*RPPH1* ratio before surgery (Fig. 4d). The Z score for the plasma *HER2*-to-*RPPH1* ratio increased gradually, and a Z score higher than the cut-off value was found on tumour relapse as multiple para-aortic lymph node metastases detected by CT. These metastatic lymph nodes were treated with trastuzumab and capecitabine plus cisplatin, after which a partial response to the treatment was achieved.

Discussion

Intratumour heterogeneity and incomplete membrane staining patterns in *HER2* testing make diagnosing *HER2* status more difficult in GC than in BC [24, 25]. *HER2* status in GC is determined on the basis of endoscopically biopsied and/or surgically resected specimens, although the heterogeneity of genomic evolution over time has not been considered for trastuzumab treatment. Because solid tumours are known to comprise various subclones, which can show different genomic evolution over time and different responses to treatment [26], *HER2* status in GC can change during progression and/or disease recurrence. In BC, the presence of *HER2*-positive tumour cells in

recurrent cancer patients with *HER2*-negative primary tumours has been reported [27]. To overcome these difficulties, in this retrospective pilot study, we investigated the possible utility of plasma cfDNA as an additional source for detecting *HER2* amplification and as additive information for predicting the therapeutic effect of trastuzumab treatment in patients with GC.

We have shown that analysing genomic DNA with rqPCR had a high accuracy to determine the *HER2* status of tumours and plasma in patients with GC, although our preliminary results need to be validated using larger cohorts of GC patients. First, to circumvent the effects of germline variations and somatic changes on the CN stability of a reference gene, we assessed candidates for an *HER2* rqPCR assay appropriate for GC. Several reference genes for PCR-based plasma *HER2* CN analyses were previously used for BC [7, 9, 11, 20]. However, the unaltered chromosomal regions can be different between GC and BC. In addition, recent studies demonstrated that polysomy 17 could affect *HER2* testing and that some BC patients with polysomy 17 should also be considered for therapy with trastuzumab [28–30]. On the basis of previous reports [11, 20–22], we selected three candidates, two on the long arm of chromosome 17 (*TKO1* and *EFTUD2*) and one on another chromosome (*RPPH1*). Among them, the CNs of the *RPPH1* gene appeared to be the stablest in GC cell lines, GC non-tumour tissues and normal plasma samples, suggesting that *RPPH1* is an appropriate

Fig. 4 Detailed analysis of the clinical histories of patients whose plasma *HER2* amplification status was monitored repeatedly before and after surgery. **a** Correlation between tissue *HER2*-to-*RPPH1* CN ratios and Z scores for plasma *HER2*-to-*RPPH1* CN ratios for 39 patients with GC. Circles represent patients with *HER2*-positive tumours and crosses represent patients with *HER2*-negative tumours. Patients with Z scores for plasma *HER2*-to-*RPPH1* CN ratios (y-axis) of more than 10 and tissue *HER2*-to-*RPPH1* CN ratios (x-axis) of more than 20 were excluded from this scatter plot. **b** Clinical course of patient MK489 with plasma *HER2* status monitoring. A 55-year-old female patient underwent surgery for GC of pathological stage T4, N3, M0, stage IIIC and an IHC *HER2* score of 3. Although postoperative abdominal lymphatic recurrence (size 16.5 mm × 7.6 mm, shape round) was detected by computed tomography (CT) at 3 months after surgery, a partial response to trastuzumab therapy was achieved at 2 months after starting treatment (size 15.8 mm × 5.0 mm; shape flat). Her plasma *HER2* CN was high before surgery, and a decreased level was maintained during treatment with trastuzumab. **c** Clinical course of patient MK412 with plasma *HER2* status monitoring. A 64-year-old male patient

underwent surgery for GC of pathological stage T3N2M0, stage IIIA and an IHC *HER2* score of 2 and negative FISH result. Because his *HER2* status was negative, anticancer drugs other than trastuzumab were used for adjuvant chemotherapy. Although his plasma *HER2* CN remained at a low level until relapse as multiple hepatic metastases, it increased dramatically after this recurrence. **d** Clinical course of patient MK418 with plasma *HER2* status monitoring. A 52-year-old male patient underwent surgery for GC of pathological stage T4N3M0, stage IIIC and an IHC *HER2* score of 3. His plasma *HER2* CN was low before surgery, but continuously increased after surgery. After lymphatic recurrence had occurred, trastuzumab therapy was initiated and the plasma *HER2* CN declined. Although a partial response to trastuzumab therapy was achieved (30.4 % reduction) once, renal failure progressed and treatment with this anticancer drug was discontinued. Increases in the sizes and numbers of metastatic lymph nodes were observed along with a slight elevation in plasma *HER2* CN. CDDP cisplatin, CPT-11 irinotecan, DOC docetaxel, H trastuzumab, LN lymph node, ND none detected, S-1 tegafur, gimeracil and oteracil potassium combination, XP capecitabine plus cisplatin

Table 3 Results of tissue and plasma real-time quantitative polymerase chain reaction (rqPCR) *HER2*-to-*RPPH1* copy number (CN) analyses in 13 patients treated with trastuzumab from development cohort

Patient ^a	Stage ^b	Tumour <i>HER2</i> status ^c		rqPCR <i>HER2</i> -to- <i>RPPH1</i> CN ratio ^d		Therapeutic effect of trastuzumab
		IHC	FISH	Tumour (RQ)	Plasma (Z score)	
MK418	III	3	–	1.36	–0.25	PR
MK749	III	3	–	2.47	–0.14	SD
MK592	III	2	Negative	NA	0.28	PR
MK426	III	3	–	2.13	1.08	SD
MK377	II	2	Positive	1.52	1.24	SD
MK614	III	3	–	39.71	2.14	PD
MK618	III	3	–	3.39	3.05	PR
MK564	IV	3	–	NA	3.07	PR
MK714	III	3	–	1.48	5.49	PR
MK489	III	3	–	10.07	6.29	PR
MK506	III	2	Positive	1.82	6.88	PR
MK694	III	3	–	18.51	7.19	PR
MK624	III	3	–	22.1	33.81	PR

Amplification-positive results are given in bold.

NA not available, PD progressive disease, PR partial response, RQ relative quantitation, SD stable disease

^a Trastuzumab therapy for patients with *HER2*-positive gastric cancer was performed on the basis of the European Medicines Agency criteria, except for patient MK592.

^b Disease stage was defined in accordance with the Union for International Cancer Control seventh tumour–lymph node–metastasis (TNM) classification using surgical-pathologic findings.

^c Tumour *HER2* status was determined by an immunohistochemistry (IHC) score 3+ or an IHC score 2+ with a positive fluorescent in situ hybridization (FISH) result as described in “Materials and Methods”.

^d Tumour and plasma *HER2* amplifications were determined by the rqPCR *HER2*-to-*RPPH1* CN test as described in “Materials and methods”.

reference for rqPCR in GC. Unlike BC [20], using a control probe from a chromosome other than chromosome 17 may provide optimal characteristics for *HER2* CN analysis in aneuploid GC cancers for which highly accurate discrimination in CN is required.

No significant association between the plasma *HER2* level and tumour *HER2* CN status determined by FISH has been reported previously [8]. In this study, however, the Z scores of plasma *HER2*-to-*RPPH1* ratios were concordant with the routine-method-based tumour *HER2* status, although this degree of concordance was lower than that in BC [20]. High Z scores for plasma *HER2*-to-*RPPH1* ratios were significantly associated with tumour size among all 43 patients and even among 13 patients with positive tissue *HER2* status. Among 13 trastuzumab-treated patients, a partial response to therapy was achieved in all seven patients with plasma *HER2* amplification, whereas a less effective response (stable disease or progressive disease) was observed in four of six patients without plasma *HER2* amplification. No relationship was found between a response to trastuzumab treatment and rqPCR-based tumour *HER2* amplification status. Therefore, we hypothesize that plasma rqPCR-based *HER2* CN analyses and routine-method-based and/or rqPCR-based tumour *HER2*

status have different meanings when used as tools for predicting a therapeutic effect in GC treatment. Because GC tumours, most of which comprise *HER2*-positive and aggressive tumour cells, could show good responses to molecular targeted therapy for *HER2*, plasma *HER2* CN testing may have clinical utility for predicting GC responsiveness to *HER2*-targeted therapy.

In this pilot study, we assessed a possible advantage of our plasma rqPCR *HER2* CN testing for monitoring *HER2* status of GC tumours in a few patients with repeated measurements over time, although the number of patients is insufficient to draw conclusion. In the *HER2*-positive patient MK418, with a low plasma *HER2* CN level before surgery (Fig. 4d), changes in plasma *HER2* CN level and clinical course during adjuvant therapy suggested that *HER2*-amplified GC cells with metastatic potential may not have composed most of the resected tumours, and this potential allowed the emergence of *HER2*-positive cells. In the *HER2*-negative patient MK412, with a concordant low plasma *HER2* CN level before surgery (Fig. 4c), a continuous increase in plasma *HER2* CN level with metastatic recurrence notwithstanding chemotherapy suggested that *HER2*-amplified GC cells having metastatic potential and resistance to chemotherapeutic reagents may have existed

as a minor population in primary tumours or that GC cells acquired *HER2* amplification and these phenotypes during tumour progression, and this potential allowed the emergence of *HER2*-positive cells. It will be interesting to ascertain the reasons for the discordance between *HER2* status in biopsied and/or resected tumours and plasma *HER2* CN status during the clinical course in a large cohort of patients with GC.

The ‘liquid biopsy’ method presented in this study using rqPCR as a repeated, non-invasive approach has several advantages for clinical applications [12]. First, a non-invasive blood-based assay is attractive for repeated sampling to evaluate tumour genomic evolution. Second, this method appears to be useful for monitoring therapeutic efficacy and recurrence as a complement to conventional serum tumour markers, such as carcinoembryonic antigen and cancer antigen 19-9, in *HER2*-positive patients with GC. Another possible application is predicting sensitivity to trastuzumab-based chemotherapy. Our plasma-based *HER2* rqPCR testing, rather than tumour *HER2* amplification levels [31], may be used to predict the sensitivity to trastuzumab-based combination chemotherapy. In addition, recurrent disease in routine clinical practice is frequently not biopsied when such a biopsy is technically challenging and there are no diagnostic doubts that relapse has occurred. A non-invasive test to screen for acquiring *HER2* amplification in these circumstances would be clinically useful.

In conclusion, our preliminary, retrospective study has demonstrated the potential clinical use of circulating cfDNA to detect *HER2* amplification by rqPCR as a therapeutic biomarker in patients with GC. The results obtained using cfDNA did not always correlate with those for a primary tumour, at least partly owing to the spatial and temporal intrinsic heterogeneity of *HER2* expression/CN status during GC progression. Further prospective examinations that would focus on the significance of a plasma *HER2* test as a non-invasive approach in advanced GC for the purpose of better molecular prediction of therapeutic effects and treatment monitoring during the clinical course of GC will be necessary.

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