SHORT COMMUNICATION

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Droplet digital PCR measurement of HER2 in patients with gastric cancer

H Kinugasa*[,1], K Nouso[1,2], T Tanaka[3], K Miyahara[1], Y Morimoto[1], C Dohi[1], T Matsubara[4], H Okada[1] and K Yamamoto[1]

[1]*Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan;* [2]*Department of Molecular Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan;* [3]*Department of Pathology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan and* [4]*BioRepository/BioMarker Analysis Center, Innovative Clinical Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan*

Background: Although there are some new criteria for human epidermal growth factor receptor 2 (HER2) expression with immunohistochemistry/fluorescence *in situ* hybridisation (IHC/FISH) in gastric cancer, the method is still ambiguous and is somewhat dependent on the subjective qualities of the evaluator.

Methods: We used droplet digital polymerase chain reaction (ddPCR) to evaluate HER2 amplification in formalin-fixed and paraffin-embedded (FFPE) samples and cell-free serum circulating tumour DNA (ctDNA) in 25 patients with gastric cancer.

Results: The concordance rate of HER2 amplification examined in FFPE samples with ddPCR and IHC/FISH was 92% (23 out of 25). The concordance rate of FFPE with ctDNA was not high (62.5%); however, patients who were HER2-positive by ctDNA had significantly shorter survival compared with HER2-negative patients.

Conclusions: Our results demonstrated that this ddPCR method was as effective as IHC/FISH and therefore might become a standard method for analysing not only FFPE but also ctDNA.

The International Agency for Research on Cancer's GROBOCAN 2012 project reported that gastric cancer is the fifth most commonly diagnosed cancer and the third most common cause of cancer-related death. Surgical resection is the mainstream treatment that can cure patients at an early stage, but the survival of patients with advanced gastric cancer treated with palliative chemotherapy remains low (Cunningham *et al*, 2006).

Recently, trastuzumab has been considered a new standard treatment option for patients with human epidermal growth factor receptor 2 (HER2, also known as ERBB2)-positive advanced gastric or gastro-oesophageal junction cancer (Bang *et al*, 2010). Evidence from several reports indicates that HER2 is an important biomarker and a key driver of tumourigenesis in gastric cancer; this finding is similar to previous reports that established HER2 as a treatment target in breast cancer (Slamon *et al*, 2001; Smith *et al*, 2007; Gravalos and Jimeno, 2008). Trastuzumab in combination with oral fluoropyrimidine plus cisplatin showed promising antitumour activity in Japanese patients (Kurokawa *et al*, 2014).

The assessment of HER2 status usually involves immunohistochemistry (IHC; Hercep Test, Dako, Denmark) and fluorescence *in situ* hybridisation (FISH; HER2 FISH pharmDx, Dako) of tumour samples. However, the HER2 evaluation of gastric tumours has some limitations owing to the inherent biological differences between gastric and breast tissue (Ruschoff *et al*, 2012). In gastric tumours, HER2 overexpression occurs in 7–34% of samples (Hofmann *et al*, 2008). However, the distribution of HER2-expressing cells is known to be heterogeneous in gastric cancer, and the staining sites in cells are irregular (Ruschoff *et al*, 2012). Although there are some new criteria for HER2 expression in gastric cancer (Hofmann *et al*, 2008), the method is still ambiguous and is somewhat dependent on the subjective qualities of the evaluator.

*Correspondence: Dr H Kinugasa; E-mail: gyacy14@yahoo.co.jp

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Digital polymerase chain reaction (PCR) provides a new method to improve the diagnostic ability and to make it possible to quantitatively and objectively assess gene amplification (Vogelstein and Kinzler, 1999). It is superior to other methods, including the multiplex ligation-dependent probe amplification (MLPA) technique, in terms of quantification (Hindson *et al*, 2011; Mason and Griffiths, 2012). We used this new technology to evaluate HER2 amplification in samples collected from gastric cancer patients, and analysed its clinical utility in the detection of amplification of cell-free serum circulating tumour DNA (ctDNA). This technique could represent a new noninvasive method to measure gene amplification.

MATERIALS AND METHODS

Patients. Biopsy tissue and serum samples collected from 25 consecutive patients with non-resectable gastric cancer who were treated between April 2011 and August 2013 were analysed in this study. These samples were obtained before the start of treatment. The median age was 66 (range: 29–81) years, and the male/female ratio was 4.0 (20/5). Overall, 2 and 23 patients were diagnosed with stage III and stage IV cancer, respectively. IHC/FISH showed that 8 patients were positive for HER2. Titanium silicate (TS)-1 (TS-1: 80 mg m^{-2}, days 1–21) and cisplatin (CDDP: 60 mg m^{-2}, day 8), irinotecan (CPT-11: 100 mg m^{-2}, day 1 and day 15), or fluorouracil (5FU: 600 mg m^{-2}, day 1) were selected as the initial chemotherapy agents. All eight patients with HER2 overexpression received trastuzumab (8 mg kg^{-1}) simultaneously with the chemotherapy agents described above. We collected 25 healthy serum samples and 25 healthy gastric tissue samples as noncancer samples for use as a reference. Overall survival was calculated from the day of diagnosis by gastric biopsy to death or the last follow-up examination. All patients provided written consent to examine their serum and to use their clinical data. Healthy donors also consented to their participation in this study. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the Okayama University Ethics Committee.

Extraction of FFPE DNA. Formalin-fixed paraffin-embedded (FFPE) samples were obtained by endoscopic biopsy. Five biopsy samples from the tumour were investigated per patient. Histological examinations confirmed that each section contained at least 30% tumour cells. We extracted DNA from five 5-μm-thick sections from the FFPE samples. DNA was extracted and purified with the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Extraction of serum ctDNA. Blood samples were collected in tubes (vacutainer #367819, BD, Franklin Lakes, NJ, USA) and processed within 1 h after collection. The samples were centrifuged at 3000 **g** at 4 °C to separate the serum from the peripheral blood cells, and they were stored at − 80 °C. ctDNA was extracted from aliquots (1 ml) of serum obtained from 5 ml of blood with the use of the QIAamp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions.

Digital PCR. Droplet digital polymerase chain reaction (ddPCR; QX200, Bio-Rad, Hercules, CA, USA) was used in this study. Each sample was partitioned into 20 000 droplets, with target and background (reference) DNA randomly, but uniformly, distributed among the droplets. The following primers were used for ddPCR: HER2 forward (5′-ACAACCAAGTGAGGCAGGTC-3′), HER2 reverse (5′-GTATTGTTCAGCGGGTCTCC-3′), MGB probe (FAM) for HER2 (5′-FAM-CCCAGCTCTTTGAGGACAAC-MGB-3′), EFTUD2 forward (5′-GGTCTTGCCAGACACCAAAG-3′), EFTUD2 reverse (5′-TGAGAGGACACACGCAAAAC-3′), and MGB probe (VIC) for EFTUD2 (5′-VIC-GGACATCCTTTG GCTTTTGA-MGB-3′). The reactions were performed in 20-μl reaction volumes that consisted of up to 10 ng of extracted DNA (5 μl), 2 × ddPCR supermix for probe (10 μl), HER2 forward primer (0.2 μl), HER2 reverse primer (0.2 μl), HER2 FAM probe (0.5 μl), EFTUD2 forward primer (0.2 μl), EFTUD2 reverse primer (0.2 μl), EFTUD2 VIC probe (0.5 μl), and deionised distilled water (3.2 μl). The emulsified PCR reactions were run in a 96-well plate on a C1000 Touch thermal cycler. The plates were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and a 10-min incubation at 98 °C. The plates were read on a Bio-Rad QX200 droplet reader using the QuantaSoft v1.4.0 software provided by Bio-Rad to assess the number of droplets positive for HER2 and/or EFTUD2. HER2 amplification with ddPCR was defined as the HER2 ratio by calculating HER2/EFTUD2 (Gevensleben *et al*, 2013). In this study, we used the EFTUD2 site as a reference for the amplification, because it is located in the same region on chromosome 17q21. 31 and was reported to have a highly stable copy number ratio with the ERBB2 locus (Gevensleben *et al*, 2013).

Statistical analysis. Survival curves were calculated using the Kaplan–Meier method. The log-rank test was used to compare the survival curves. The Wilcoxon signed rank test and the chi-squared test were used for continuous variable data and categorical data, respectively. Differences with *P*-values less than 0.05 were considered statistically significant. All analyses were performed with the JMP statistical software (ver. 9.0.0 SAS institute, Japan).

RESULTS

Setting a cutoff value for digital PCR. We confirmed HER2 gene amplification status in twenty-five healthy gastric tissue samples and twenty-five healthy serum samples before this assessment. The median HER2 ratio of the tissue samples was 0.25 (range: 0.18–0.53), whereas the median HER2 ratio of the serum samples was 1.05 (range: 0.51–1.14). We set the HER2 cutoff value at 1.2, because the value of all healthy serum and tissue samples was below 1.2. Gevensleben *et al* set the HER2 cutoff value at 1.25 in breast cancer samples (Gevensleben *et al*, 2013); therefore, these data support the validity of our HER2 cutoff value.

HER2 expression and copy number in tissues. HER2 criteria (IHC/FISH score) have five scores: 0, 1+, 2+/FISH negative, 2+/FISH positive, or 3+. An IHC score of 2+ indicates equivocal staining, with FISH performed to confirm the HER2 status. Scores of 0, 1+, or 2+/FISH negative are negative for HER2, whereas 2+/FISH positive or 3+ are positive for HER2 (Ruschoff *et al*, 2012). Overall, 17 and 8 patients were HER2-negative and HER2-positive by IHC/FISH, respectively. In addition, HER2 copy number in DNA from FFPE was measured with ddPCR. The concordance rate of HER2 amplification from the DNA samples and IHC/FISH was 92% (23 out of 25) (Table 1).

Table 1. HER2 status in FFPE with ddPCR compared with IHC/FISH

	HER2 with IHC/FISH	
HER2 in FFPE with ddPCR	Positive	Negative
Positive	7	1
Negative	1	16

Abbreviations: ddPCR = droplet digital polymerase chain reaction; FFPE = formalin-fixed paraffin-embedded; FISH = fluorescence *in situ* hybridisation; HER2 = human epidermal growth factor receptor 2; IHC = immunohistochemistry.

HER2 copy number in serum ctDNA. HER2 was also measured with ddPCR in serum ctDNA in 24 out of 25 patients. We could not examine ctDNA in the sample from one patient because of shortage of serum. The median HER2 ratio (HER2/EFTUD2) in the ctDNA was 1.15 (range: 0.94–8.4). If we defined a ratio >1.2 as HER2-positive and ratios below this value as negative, 7 and 17 patients were positive and negative, respectively. Although the positivity of HER2 in ctDNA was similar to that obtained by IHC/FISH (7 out of 24 *vs* 8 out of 25), the concordance rate of FFPE with ddPCR was 62.5% (15 out of 24) (Table 2).

There was no significant difference with regard to survival between patients whose samples were HER2-negative and HER2-positive via ddPCR on FFPE or IHC/FISH (Figure 1). Interestingly, patients with HER2-positive status determined by ctDNA showed significantly shorter survival period compared with HER2-negative patients. The median survival times of HER2-positive and HER2-negative patients were 124 days and 321 days, respectively ($P=0.01$) (Figure 1).

Age, sex, tumour stages, and tumour histology were not significantly different between the patients who were HER2-positive or HER2-negative based on ctDNA analysis (Table 3).

DISCUSSION

HER2 is a member of a receptor family associated with tumour cell proliferation, apoptosis, adhesion, migration, and differentiation (Slamon *et al*, 2001). Although some studies have reported that HER2 overexpression in gastric cancer is associated with poor outcomes and aggressive disease (Tanner *et al*, 2005), the relationship between HER2 expression and the prognosis of gastric cancer patients has not been elucidated.

Table 2. HER2 as assessed by ddPCR in ctDNA compared with FFPE

	HER2 in FFPE with ddPCR	
HER2 in ctDNA with ddPCR	**Positive**	**Negative**
Positive	3	4
Negative	5	12

Abbreviations: ctDNA = circulating tumour DNA; ddPCR = droplet digital polymerase chain reaction; FFPE = formalin-fixed paraffin-embedded; HER2 = human epidermal growth factor receptor 2.

Our study demonstrated that ddPCR is a useful method for evaluating HER2 status in FFPE samples; this technique may be useful as an alternative to IHC/FISH, although confirmatory studies are necessary. The most valuable aspect of this method is that it enables objective evaluation because it provides numerical values. Conventional methods depend on the subjective evaluation of images, but this technique allows the user to digitalise HER2 status. Furthermore, we showed the possibility of measuring ctDNA with ddPCR to determine HER2 status. The concordance rate for HER2 detection by ddPCR between FFPE and ctDNA was not high in the present study, but it is interesting that HER2 in ctDNA may have potential as a predictive factor.

We examined the difference in characteristics between patients with HER2-positive and HER2-negative serum samples. However, no clear difference was observed, except that HER2-positive patients tended to have intestinal type gastric cancer, which has been previously reported as a characteristic of HER2-positive tissues (Ruschoff *et al*, 2012). In addition, we observed shorter survival in patients with HER2-positive sera than in patients with

Table 3. The distribution of prognostic factors between HER2-positive and HER2-negative ctDNA samples

	HER2-positive in ctDNA	HER2-negative in ctDNA	*P*-value
Number of patients	7	17	
Age (years, median (range))	67 (60–76)	64 (29–81)	0.464
Sex			
Male	6	13	0.612
Female	1	4	
Tumour stage			
III	0	1	0.512
IV	7	16	
Histology			
Intestinal type	5	7	0.177
Diffuse type	2	10	
HER2 with IHC/FISH			
Positive	4	4	0.112
Negative	3	13	

Abbreviations: ctDNA = circulating tumour DNA; FISH = fluorescence *in situ* hybridisation; HER2 = human epidermal growth factor receptor 2; IHC = immunohistochemistry.

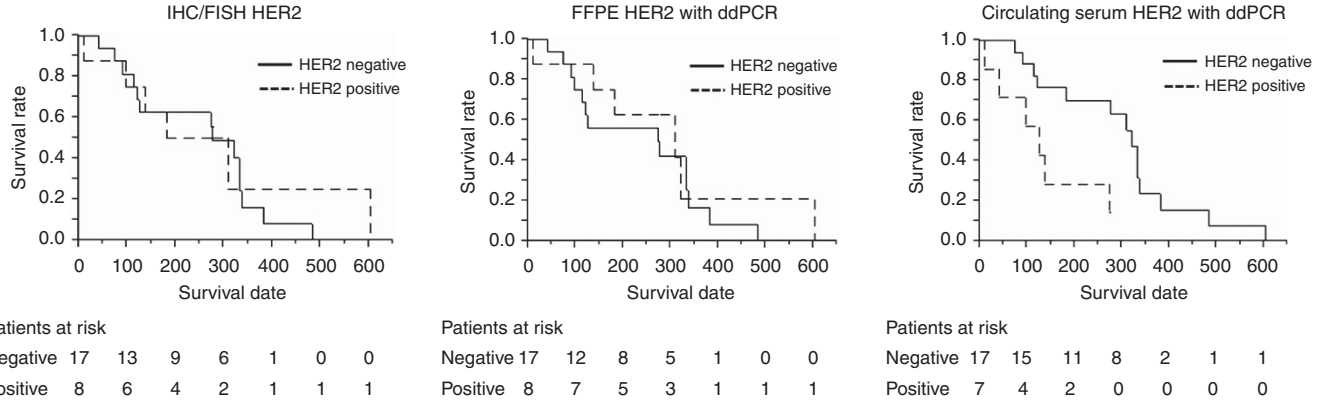

Figure 1. The survival rates for HER2-negative (solid line) and HER2-positive (dotted line) patients (as determined by IHC/FISH) were not significantly different (median survival times (MSTs) were 275 and 245 days for HER2-negative and HER2-positive patients, respectively). FFPE assessments showed that the survival rates were not significantly different (MSTs of 273 and 309 days for HER2-negative and HER2-positive patients, respectively). According to ctDNA results, the survival rates were significantly different ($P=0.01$, MSTs for HER2-negative and HER2-positive patients of 321 and 124 days, respectively).

HER2-negative sera; however, no difference was observed between patients with HER2-positive tissue samples and those with HER2-negative tissue samples. The appearance of ctDNA might indicate rapid tumour turnover, suggesting that the HER2-positive cancers with ctDNA have higher malignant potential than HER2-positive cancers without ctDNA.

Two out of 25 patients exhibited inconsistent HER2 results between the two methods. This type of discrepancy between IHC/FISH and ddPCR is similar to the discrepancy reported between IHC and FISH (Sauter *et al*, 2009; Bang *et al*, 2010). For example, the discrepancy reported in the ToGA trial was 2–13% (Bang *et al*, 2010). These discrepancies may be owing to the preservation status of the samples or expression differences between the DNA and protein levels.

The median HER2 ratio of healthy tissue samples was lower than that of healthy serum samples. This discrepancy might be owing to differences in the quality of the extracted DNA from the FFPE and serum samples. Another possibility is that there was an influx of DNA with different HER2 ratios into the sera from other tissues. We adopted the same cutoff value for serum samples (1.2) and tissue samples, because no clear difference was observed, even when we examined the tissue samples with a different cutoff value that represented the upper limit of the healthy tissue samples (0.6).

Other studies have attempted to detect rare mutations in plasma ctDNA (Dawson *et al*, 2013; Murtaza *et al*, 2013). However, whether such mutations can be detected more reliably in serum or plasma samples is unclear (Gormally *et al*, 2007). In this study, we used serum to detect HER2 gene amplification. Our preliminary trial conducted before the present study showed that there was no difference between the detection rates of mutations in serum and plasma (data not shown).

The technical limitations of ddPCR are that the specific target is limited and this procedure is not a one-step method because of the requirement to generate droplets (Hindson *et al*, 2011). Another limitation is that we did not use the microdissection method to obtain cancer cells from the FFPE samples. The employment of this method is more efficient when the absolute copy number is known, although samples with tumour content as low as 30% are sufficient for the detection of the amplification.

In conclusion, this ddPCR method was able to digitise HER2 status, and it might become a standard method for analysing not only FFPE but also ctDNA. A large-scale prospective study with the design controls for use of trastuzumab is needed to affirm the utility of this assay for the diagnosis and the prediction of prognosis.

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