Monitoring gastric cancer progression with circulating tumour DNA

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Background: Circulating tumour DNA (ctDNA) is an emerging candidate biomarker for malignancies and may be useful for monitoring the disease status of gastric cancer.

Methods: We performed targeted deep sequencing of plasma cell-free DNA (cfDNA) by massively parallel sequencing in patients with tumours harbouring *TP53* mutations. The quantitative values of *TP53*-ctDNA during the clinical course were compared with the tumour status.

Results: Three out of ten patients with *TP53* mutations in primary tumours showed detectable *TP53* mutation levels in preoperative cfDNA. Although the cfDNA concentrations were not always reflective of the disease course, the ctDNA fraction correlated with the disease status.

Conclusions: ctDNA may serve as a useful biomarker to monitor gastric cancer progression and residual disease.

Computed tomography (CT) is the major diagnostic tool for clinical management of gastric cancer. However, minimal residual disease and peritoneal dissemination, the most frequent recurrent patterns in gastric cancer, are typically difficult to detect with CT. Circulating tumour DNA (ctDNA)—fragmented DNA released from cancer cells into the bloodstream—is an emerging candidate marker to follow disease progression (Schwarzenbach *et al*, 2011). Previous studies have indicated that ctDNA levels correlate with tumour burden in colon cancer (Diehl *et al*, 2008) and breast cancer (Dawson *et al*, 2013). However, no comparative studies have been conducted in gastric cancer, and it is necessary to examine the possibility that ctDNA can be used for monitoring disease status, especially minimal residual disease and peritoneal dissemination. We previously developed a system to detect *EGFR* mutations in the plasma of lung cancer patients using a massively parallel sequencer (Kukita *et al*, 2013) and applied it to monitor advanced gastric cancer.

MATERIALS AND METHODS

Sample collection. Gastric cancer tissue and blood were prospectively collected from 42 patients who underwent gastrectomy for advanced gastric cancer at the Osaka University Hospital from April 2011 to November 2012. Tumour tissue was obtained from the primary lesion of the resected specimen immediately after surgery and stored at $-80\,^\circ\text{C}$ as a fresh-frozen sample. Peripheral blood (5 ml) was drawn using an EDTA-2Na tube at admission (before surgery), before discharge (after surgery), and, in three patients, after recurrence. After removal of cellular debris and platelets by centrifugation, the plasma samples were stored at $-80\,^\circ\text{C}$ until DNA extraction. Written informed consent for this study was obtained from all patients. The study was approved by the Ethics Committees of the Osaka University and the Osaka Medical Center for Cancer and Cardiovascular Diseases.

Mutation detection in the primary tumours. DNA was extracted using a DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, using serial 40-nm sections. *TP53* exons 4–10 were amplified by PCR (Supplementary Table 1), and direct sequencing was performed with an ABI3730xl/ABI3100 using BD v3.1, following the EXO-1/SAP procedure.

Mutations in other genes were searched using Ion AmpliSeq Cancer Panel, version 1 (Life Technologies, Carlsbad, CA, USA),

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targeted for 739 hot-spot mutations in 604 loci in the 46 cancer-related genes, following the supplier's protocol.

Deep sequencing of the amplified target regions. Cell-free DNA was extracted from 1.2 ml of plasma using a Circulating Nucleic Acids Kit (Qiagen). DNA was eluted in a final volume of 40 μl. A volume of 10 μl was used for a single sequencing reaction. DNA concentrations were measured using a method based on LINE-1 copy number (Rago *et al*, 2007). The PCR primers were designed to generate final products ~ 100 base pairs in length (Supplementary Table 2).

TP53 target regions were amplified from patient cell-free DNA or genomic DNA purified from the white blood cells obtained from normal individuals. They were then sequenced using a semiconductor sequencer as previously described (Kukita *et al*, 2013), maintaining the number of reads as $> 100\,000$. Data analysis was performed as described.

RESULTS

Selection of patients for the assessment of gastric cancer progression. Given that gastric cancer somatic mutations occur most frequently in *TP53*, we chose *TP53* as the target gene. Direct sequencing of the *TP53* gene revealed that 10 (24%) of the 42 tumours analysed harboured mutations (Supplementary Table 3). Because cell-free DNA was available before surgical resection in 6 of these 10 cases, we performed cell-free DNA deep sequencing to evaluate the mutations identified in each primary tumour (Supplementary Figure 1, Supplementary Table 4). Given that one plasma sample corresponding to 1 ml of whole blood typically includes cell-free DNA corresponding to ~ 5000 genomes deduced from the mean concentration in our lung cancer study (Kukita *et al*, 2013), base changes of $< 0.02\%$ are considered background. Mutations in three of the six cases fulfilled this criterion. It may be noted that the number of reads with base changes of excluded cases did not exceed that corresponding to a single molecule estimated from the amount of DNA used for sequencing.

Detection threshold values were based on deep sequencing data from 24 normal samples as described previously (Kukita *et al*, 2013). We used DNA purified from white blood cells, because the error profile of white blood cells was not substantially different from that of cell-free DNA in our previous study. The threshold values obtained for base changes in 100 000 reads were as follows: 603 for c.103delT (patient 4), 11 for c.747G$>$C (patient 5), and 39 for c.166G$>$T (patient 8; Supplementary Table 5). Because the initial levels of all three cases exceeded the threshold, these cases were used for the follow-up study.

Comparison of cell-free DNA and ctDNA as indicators of disease progression. The median concentration of extracted cell-free DNA of all samples from the three cases was 43.1 ng ml^{-1} plasma (range 9.5–1338 ng ml^{-1}). The mutant fraction range was 0.001–77.8% (median 0.90%).

First, we compared cell-free DNA and ctDNA dynamics for the ability to monitor the treatment response (Figure 1). The cell-free DNA values fluctuated in all but one of the patients, regardless of the disease course. In one patient (patient 8), cell-free DNA increased along with the rapid progression of multiple liver metastases. In this patient, the majority of the cell-free DNA was derived from the tumour. By contrast, ctDNA dynamics were concordant with disease progression.

ctDNA validation using additional mutant genes. For the three cases, mutations in genes other than *TP53* were surveyed with the Ion AmpliSeq Cancer Panel. The results are shown in Supplementary Table 6. We focused on *PIK3CA* c.1633G$>$A (found in patients 4 and 5) and *FBXW7* c.1177C$>$T (found in patient 8) and confirmed these mutations by direct sequencing (Supplementary Table 1). We analysed the same plasma samples, which were subjected to *TP53* analysis, by deep sequencing of the new genes. ctDNA fraction values estimated from the assay
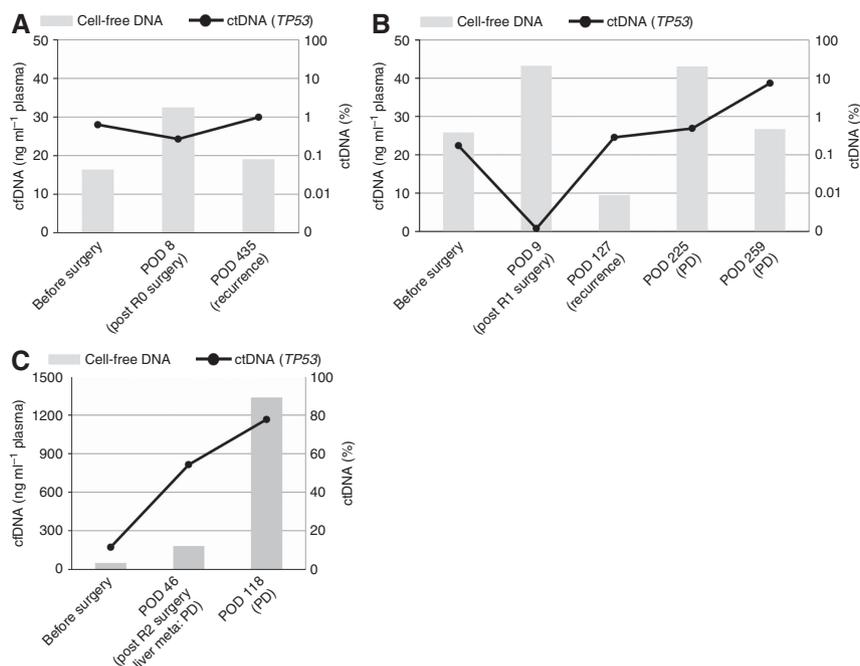
Figure 1. Comparison of cell-free DNA and ctDNA with regard to treatment response. The levels of cell-free DNA (cfDNA; grey bar) and ctDNA (line graph) at various time points in patients 4 (**A**), 5 (**B**), and 8 (**C**) are displayed. The vertical axis on the left represents the concentration of cfDNA, and the vertical axis on the right represents the *TP53*-mutant fraction in cfDNA. The horizontal axis labels represent the time points of plasma sampling and tumour status (in parenthesis). The presence or absence of tumour after surgery is described by the R status (R0: no residual tumour, R1: microscopic residual tumour, R2: macroscopic residual tumour). Abbreviations: POD: postoperative day; PD: progressive disease.
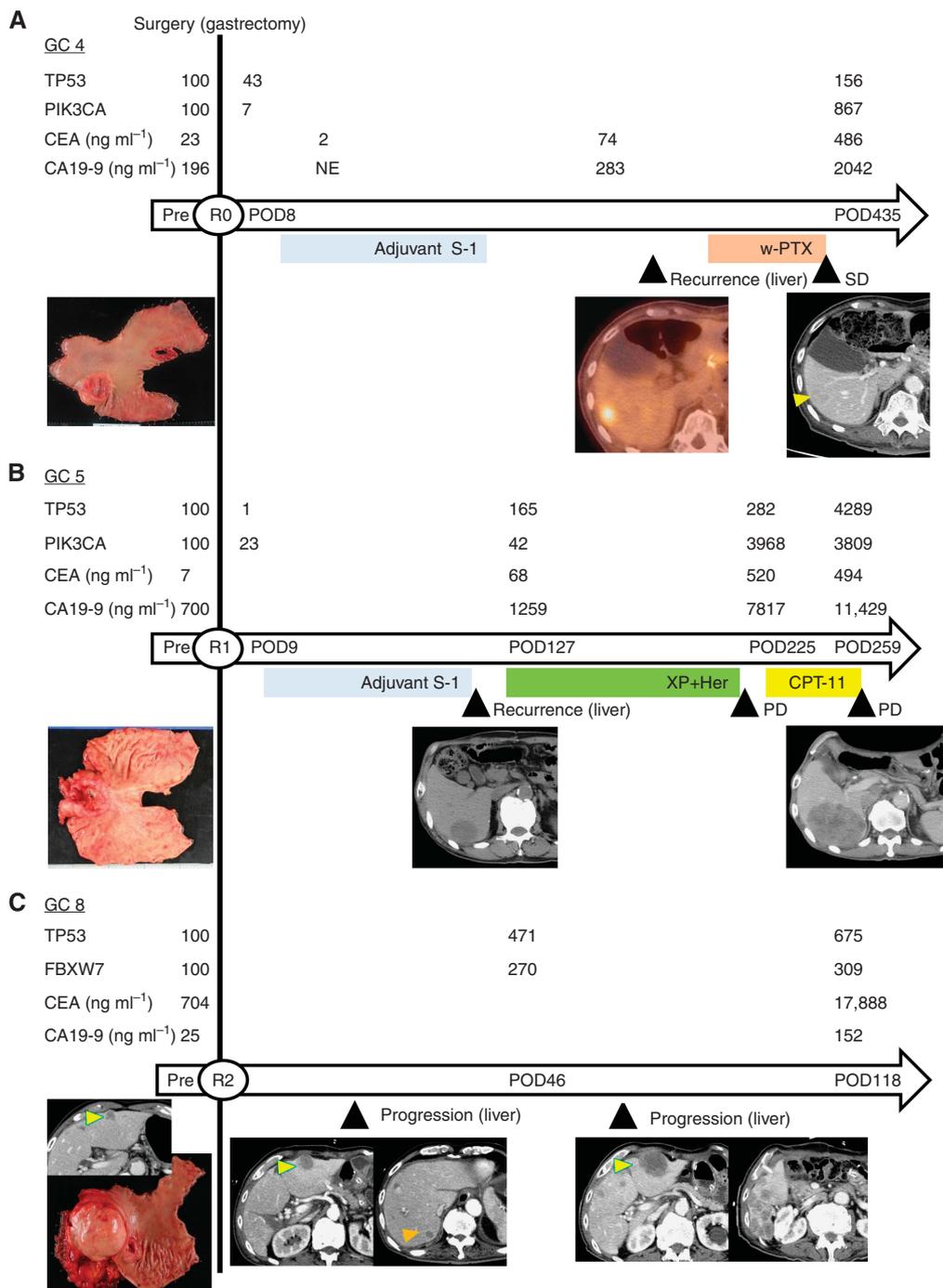
Figure 2. The relationship between ctDNA and clinical observations during the treatment course. ctDNA values during the clinical course are depicted in comparison with serum tumour markers, treatment, response, and tumour burden (specimen photos and radiological images). The horizontal arrows indicate the time scale for each case. The day of gastrectomy is represented by the vertical line. The presence or absence of tumour after surgery is described by the R status (R0: no residual tumour, R1: microscopic residual tumour, R2: macroscopic residual tumour). The intervals from surgery are indicated inside the arrows. Chemotherapy is depicted by colour bars under the time scale. The specimen photos depict the resected stomach, representing the tumour burden before surgery. CT (**A**, **B**, and **C**) and positron emission tomography (PET)-CT (**A**) images display the status of metastatic liver tumours at each assessment point, indicated by triangle markers. In each case, ctDNA is expressed as the percent relative to the ctDNA values before surgery. POD: postoperative day, PD: progressive disease, XP: capecitabine + cisplatin, Her: trastuzumab, CPT-11: irinotecan, w-PTX: weekly administration of paclitaxel. (**A**) The ctDNA fraction was measured for both *TP53* (c.103delT) and *PIK3CA* (c.1633G>A). (**B**) The ctDNA fraction was measured for both *TP53* (c.747G>C) and *PIK3CA* (c.1633G>A). (**C**) The ctDNA fraction was measured for both *TP53* (c.155G>T) and *FBXW7* (c.1177C>T).

results of two different genes (*TP53* and *PIK3CA/FBXW7*) were within one order of magnitude in 8 (73%) of the 11 samples, suggesting a good dynamic concordance of the different genes (Figure 2).

The relationship between ctDNA and clinical findings during the clinical course of gastric cancer. The clinical and genetic characteristics of the three cases are summarised in Table 1. CT images were evaluated using the Response Evaluation Criteria in

Table 1. Clinical and genetic characteristics of three cases assessed by follow-up study

Patient number.	Age	Gender	Histological type	p-Stage	Residual tumour after surgery	Gene mutation 1	Gene mutation 2	Postoperative chemotherapy	Metastasis site, number, timing	Treatment for metastasis	Response[a]
4	86	Male	Differentiated	IIIC	R0	TP53 c.103delT	PIK3CA c.1633G>A	Yes	Liver, single, POD275	Chemotherapy	SD
5	73	Male	Differentiated	IV[b]	R1	TP53 c.747G>C	PIK3CA c.1633G>A	Yes	Liver, multiple, POD93	Chemotherapy molecular-target drug	PD
8	80	Male	Differentiated	IV[c]	R2	TP53 c.166G>T	FBXW7 c.1177C>T	No	Liver, multiple, (residue)	None	PD

Abbreviations: PD = progressive disease; POD = postoperative day; R1 = microscopic residual tumour; R2 = macroscopic residual tumour; R0 = no residual tumour (curative resection); SD = stable disease.
[a]According to the Response Evaluation Criteria in Solid Tumors (RECIST).
[b]Peritoneal cytology positive for carcinoma cells, and
[c]Hepatic metastasis.

Solid Tumors (RECIST; Eisenhauer et al, 2009). The alterations in the ctDNA levels and clinical disease course are presented in Figure 2. Details of individual cases are provided below.

Case 1 (patient 4). Curative distal gastrectomy was performed for a 55-mm tumour with multiple lymph node metastases, but the patient suffered from a single liver metastasis. ctDNA of *TP53* c.103delT that was 0.63% before surgery, decreased to 0.27% (below the background noise level) after surgery, and increased to 0.98% after recurrence. Similar alterations were observed in the *PIK3CA* c.1633G>A ctDNA.

Case 2 (patient 5). Distal gastrectomy was performed for a 65-mm tumour with multiple lymph node metastases. Intraoperative peritoneal cytology was positive. Despite postoperative S-1 chemotherapy, the patient developed multiple liver metastases. Recurrent tumours were treated with chemotherapy, but the disease progressed. ctDNA levels of *TP53* c.747G>C and *PIK3CA* c.1633G>A were 0.17% and 0.84%, respectively, before surgery. ctDNA levels decreased after resection and increased after recurrence and tumour progression in both the genes. However, there was some discrepancy between the two genes after the use of trastuzumab.

Case 3 (patient 8). A Stage IV gastric cancer with a 100-mm lymph node metastasis was removed by palliative gastrectomy. A 17-mm liver metastasis was left unresected. The liver tumours grew rapidly in size and number after surgery. The ctDNA levels of *TP53* c.166G>T and *FBXW7* c.1177C>T were 11% before surgery and continually increased with tumour progression, reaching 77.8%.

DISCUSSION

In this report, we demonstrated that concentrations of ctDNA, not total plasma DNA, exhibited good correlation with the disease status of gastric cancer. We observed a decrease in ctDNA levels after surgical resection, indicating its potential application for the detection of residual disease. Tie *et al* (2014) demonstrated the possibility that recurrence of stage II colorectal cancer after surgical resection may be predicted by ctDNA. Similar applications may be possible for gastric cancer cases. Serological biomarkers such as CA19-9 and CEA are used to assist in the diagnosis of CT, but they have limitations owing to their low sensitivity (Shimada *et al*, 2013). In our study, ctDNA levels were parallel with these markers, suggesting a comparable ability to monitor the disease progression. However, from our analysis, it is still unknown whether ctDNA is advantageous over serological biomarkers because of our small number of patients.

There have been several studies on cell-free DNA obtained from the blood of gastric cancer patients, using concentration (Park *et al*, 2012), *MYC/GAPDH* ratio (Park *et al*, 2009), and promoter methylation status of *MGMT*, *p15*, and *hMLH1* (Kolesnikova *et al*, 2008). These markers are not necessarily specific to cancer cells and therefore lack theoretical backgrounds for their validity. By contrast, cancer-related somatic mutations such as those in *TP53* are specific to malignancies, and the DNA with these mutations is indicative for the presence of malignancies. Our results also agree with this reasoning; levels of the somatic mutations were correlated with surgical resection and disease progression, but concentrations of cell-free DNA were not.

The recent attention to ctDNA is due mainly to technical developments, especially that of digital PCR pioneered by BEAMing (Dressman *et al*, 2003). It is important to note that massively parallel sequencers may be regarded as digital PCR because they employ digital PCR in the template preparation step after global amplification of target DNA. Because of the much higher throughput of data production than with other digital PCR technologies and the considerable investment, the massively parallel sequencers should be the method of choice if several technical problems, such as read error (Junemann *et al*, 2013), are resolved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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