Original article

Title

Circulating Tumor DNA Analysis Detects FGFR2 Amplification and Concurrent Genomic Alterations Associated with FGFR Inhibitor Efficacy in Advanced Gastric Cancer

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Running title

ctDNA analysis detects FGFR2 amplification in advanced GC

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Additional information

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Translational relevance
The efficacy of fibroblast growth factor receptors (FGFR) inhibition has not been clear for FGFR2-amplified advanced gastric cancer (GC) with significant genomic heterogeneity,
because adequate test to detect \textit{FGFR2} amplification for this population has not been established before FGFR treatment. In this study, we demonstrated that the utility of circulating tumor DNA (ctDNA) to evaluate targetable genomic alterations including \textit{FGFR2} amplification, and to guide targeted therapy in advanced GC. In addition, we also suggested that ctDNA sequencing may be useful for assessing other concurrent genomic alterations including resistance alterations for guiding the management of this type of cancer.
Abstract

Purpose:

Fibroblast growth factor receptor 2 (FGFR2) amplification is associated with poor prognosis in advanced gastric cancer (GC) and its subclonal heterogeneity has been revealed. Here, we examined whether circulating tumor DNA (ctDNA) was useful for detecting FGFR2 amplification and co-occurring resistance mechanisms in advanced GC.

Experimental Design:

We assessed genomic characteristics of FGFR2-amplified advanced GC in a nationwide ctDNA screening study. We also analyzed FGFR2 amplification status in paired tissue and plasma samples with advanced GC. In addition, we examined patients with FGFR2-amplified advanced GC identified by ctDNA sequencing who received FGFR inhibitors.

Results:

FGFR2 amplification was more frequently detected by ctDNA sequencing in 28 (7.7%) of 365 patients with advanced GC than by tissue analysis alone (2.6–4.4%). FGFR2 amplification profiling of paired tissue and plasma revealed that FGFR2 amplification was detectable only by ctDNA sequencing in 6 of 44 patients, which was associated with a worse prognosis. Two patients in whom FGFR2 amplification was detected by ctDNA sequencing
after tumor progression following previous standard chemotherapies but not by pretreatment
tissue analysis had tumor responses to FGFR inhibitors. A third patient with FGFR2 and
MET co-amplification in ctDNA showed a limitation of benefit from FGFR inhibition,
accompanied by a marked increase in the MET copy number.

Conclusions:

ctDNA sequencing identifies FGFR2 amplification missed by tissue testing in
patients with advanced GC, and these patients may respond to FGFR inhibition. The utility
of ctDNA sequencing warrants further evaluation to develop effective therapeutic strategies
for patients with FGFR2-amplified advanced GC.
Introduction

Gastric cancer (GC) remains an important cancer, being the fifth most frequently diagnosed cancer and the third leading cause of cancer death worldwide (1). Despite the advance in systemic therapy, the prognosis of patients with advanced GC is still poor with median survival time of approximately one year. Molecularly targeted therapeutic strategies have been attempted for patients with advanced GC, but have frequently failed to improve overall survival (OS) due to its nature of molecular heterogeneity (2-10).

Fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3, and FGFR4) are transmembrane receptor tyrosine kinases, and FGF/FGFR signaling can be aberrantly activated by altered FGFR genes in cancers (11). Approximately 5% of patients with GC have FGFR2 amplification (12), which is associated with poor prognosis (13-15). The relevance of high-level FGFR2 amplification in GC to the response to FGFR inhibitors has been suggested in preclinical studies (16-18); however, a randomized phase II trial (SHINE) failed to demonstrate improved progression-free survival (PFS) with the pan-FGFR tyrosine kinase inhibitor (TKI) AZD4547 compared to paclitaxel in the second-line treatment of advanced GC with FGFR2 amplification confirmed by a tissue testing (19).

The analysis of circulating tumor DNA (ctDNA) has been demonstrated to be able to detect genomic alterations in tumor cells throughout the body and has been suggested as a
method to assess heterogeneous resistance mechanisms (20,21). A translational study of patients with FGFR2-amplified advanced GC treated with AZD4547 indicated that ctDNA sequencing identified high-level FGFR2 amplifications in responders (16). A utility of ctDNA sequencing for identifying heterogeneous FGFR2 amplification within spatially distinct regions of the primary tumor and distant metastases has also been suggested (22). In addition, previous studies suggested the utility of ctDNA sequencing for identifying genomic resistance mechanisms in advanced GC harboring ERBB2, MET, and EGFR amplifications (23-26). These observations suggest that ctDNA sequencing may be useful in guiding therapy for FGFR2-amplified advanced GC by detecting FGFR2 amplification, including cases missed by single-lesion tumor biopsies, and by identifying heterogeneous resistance mechanisms. Indeed, recently, a randomized phase II trial reported that the addition of bemarituzumab, a monoclonal antibody against FGFR2b, to chemotherapy improved survival in patients with FGFR2b-overexpressing or FGFR2 amplified GC identified by tissue or ctDNA analysis (NCT03343301) (27).

Here, we evaluated the utility of ctDNA sequencing compared to tissue analysis for detecting FGFR2 amplification in advanced GC as well as other genomic alterations, including resistance alterations, and for guiding the management of this type of cancer. The ctDNA sequencing revealed that some patients with FGFR2-amplified advanced GC in
ctDNA may benefit from FGFR inhibition but cannot be identified by current tissue testing practices and clarified resistance mechanisms.
Materials and Methods

GI-SCREEN and GOZILA study design and patient selection

SCRAM-Japan GI-SCREEN is a nationwide tumor tissue cancer genomic profiling study involving 26 core cancer institutions in Japan(28), which aims to characterize the genomic landscape for all gastrointestinal cancers and accelerate development and improve care in this area by matching patients to suitable clinical trials. The key inclusion criteria included the following: i) histopathologically confirmed unresectable or metastatic gastrointestinal cancer; ii) receipt (or planned receipt) of systemic therapy; iii) age ≥20 years; iv) an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0–1; v) adequate organ function; and vi) available tumor tissue. Eligible patients provided written informed consent. The genotyping of archival or fresh tumor tissue samples from enrolled patients was performed using the Oncomine Comprehensive Assay (OCA; Thermo Fisher Scientific, Waltham, MA), which is described in more detail below. This study was initiated in February 2015 and completed enrollment in April 2019.

GOZILA is a nationwide plasma genomic profiling study in Japan based on the SCRAM-Japan GI-SCREEN platform, which, like GI-SCREEN, aims to effectively identify patients with gastrointestinal cancers who might benefit from targeted therapy, with 31 institutions including the above 26 sites(28). The key inclusion criteria were similar to those
of GI-SCREEN: i) histopathologically confirmed unresectable or metastatic GI cancer; ii) age ≥20 years; and iii) a life expectancy of at least 12 weeks. In order to avoid the suppression of ctDNA shedding due to chemotherapy, patients were included only if they showed disease progression during systemic chemotherapy and had not started the subsequent therapy at the time of blood sampling. Eligible patients provided written informed consent, and ctDNA genotyping was performed using Guardant360 (Guardant Health, Inc., Redwood City, CA), which is described in additional detail below. This study was launched in January 2018.

Both studies were conducted in accordance with the Declaration of Helsinki and the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects. Each study protocol was approved by the institutional review board of each participating institution and registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (protocol IDs UMIN000016344 for GI-SCREEN and UMIN000029315 for GOZILA).

**FGFR2 profiling concordance study design and patient selection**

A retrospective study was performed to evaluate the concordance of FGFR2 amplification between tissue and plasma in patients with advanced GC between January 2015 and December 2018 at the National Cancer Center Hospital East. Based on the
recommended FFPE-sample storage period from our previous study (29), the tissue samples collected within 4 years were used. Patients who met the following criteria were included: 1) presence of histologically confirmed gastric adenocarcinoma; 2) receipt of systemic treatment for advanced disease; and 3) an available plasma sample collected near the time of tumor biopsy and before the initiation of systemic treatment. Patients with tumors previously known to harbor FGFR2 amplification in GI-SCREEN or GOZILA who had available matched plasma and tissue samples were preferentially included. Tumor responses were assessed according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1) (30).

This study was conducted in accordance with the Declaration of Helsinki and the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects. The study protocol was approved by the Institutional Review Board at the National Cancer Center (UMIN000041008). Written informed consent was obtained from patients who were alive at the time of the study. For deceased patients and their relatives, we disclosed the study design on the website of the National Cancer Center and gave the families a chance to express the will of the decedents.
Tissue-based next-generation sequencing (NGS) analysis

For patients enrolled in GI-SCREEN and the retrospective concordance study, the NGS analysis of tumor tissue was performed using OCA v1 and OCA v3 at the Life Technologies Clinical Services Lab (West Sacramento, CA), a Clinical Laboratory Improvement Amendments (CLIA)-certified, College of American Pathologists (CAP)-accredited laboratory, as previously described (31). These assays examined 143 (OCA v1) and 161 (OCA v3) cancer-related genes and detected relevant single nucleotide variants (SNVs), copy number variations, gene fusions, and indels in one streamlined workflow. Briefly, tumor DNA and RNA were isolated from formalin-fixed paraffin-embedded sections, and DNA/RNA libraries were prepared. Purified libraries were sequenced using Ion Torrent PGM (Thermo Fisher Scientific). Sequence reads were aligned to the hg19 assembly and were called using Ion Reporter Software version 4.4 (for OCA v1) and v5.0 (for OCA v3) to detect alterations.

cTNA-based NGS analysis by Guardant360

For patients enrolled in GOZILA and the retrospective concordance study, the NGS analysis of ctDNA was performed using Guardant360 at Guardant Health, a CLIA-certified, CAP-accredited, New York State Department of Health-approved laboratory, as previously
described(32). Guardant360 detects SNVs, indels, fusions, and copy number alterations in 74 genes with a reportable range of ≥0.04%, ≥0.02%, ≥0.04%, and ≥2.12 copies, respectively. For GOZILA patients, 2 × 10-ml whole blood samples were collected from enrolled patients in Streck Cell-Free DNA blood collection tubes (BCTs) (Streck, Inc., La Vista, NE) and sent to Guardant Health. For the other patients, 3 ml of frozen plasma prepared from whole blood collected in EDTA tubes was sent for analysis. Five to 30 nanograms of cell-free DNA (cfDNA) isolated from plasma were labeled with nonredundant oligonucleotides (‘molecular barcoding’), enriched using targeted hybridization capture, and sequenced on an Illumina NextSeq 550 platform (Illumina, Inc., San Diego, CA). Base call files generated by Illumina’s RTA software version 2.12 were demultiplexed using bcl2fastq version 2.19 and processed as previously described(32). Somatic cfDNA alterations were identified using a proprietary bioinformatics pipeline.

Immunohistochemistry

FGFR2 immunohistochemistry (IHC) was performed using a rabbit anti-FGFR2 polyclonal antibody (18601; IBL, Fujioka, Japan) at Geneticlab (Sapporo, Japan), and MET IHC was performed using a rabbit anti-c-MET monoclonal antibody (790-4430; Ventana, Oro Valley, CA) at National Cancer Center (Kashiwa, Japan). FGFR2 IHC results were scored
according to the intensity and percentage of positively stained carcinoma cells, as follows: 0, no positive cells; 1, weak staining and ≥10%; 2, strong staining and <10%; 3, strong staining and 10–49%; and 4, strong staining and ≥50%.

Fluorescence in situ hybridization (FISH)

The assessment of FGFR2 amplification by FISH was conducted using an FGFR2/CEP10 probe (Geneticlab, Sapporo, Japan) (Supplementary Table 1) for twenty tumor nuclei per sample at Geneticlab (Sapporo, Japan). FGFR2 amplification was defined as an FGFR2 copy number ≥ 4.0 signals per cell and FGFR2/CEP10 ratio ≥ 2.0. If the FGFR2 copy number was ≥ 4.0 signals per cell and the FGFR2/CEP10 ratio was < 2.0, the case was defined as polysomy.

Statistical analysis

Associations of the FGFR2 status with clinicopathological factors and the variant allelic frequency (VAF) were analyzed using Fisher’s exact test or Mann-Whitney U test. OS was defined as the interval from the first day of the first-line chemotherapy to the day of death or the most recent follow-up visit. Kaplan–Meier curves were constructed, and statistical significance was determined using the log-rank test. A p-value of <0.05 was considered
significant. JMP software (ver. 14.0, SAS Institute Inc., Cary, NC, USA) was used to perform statistical analyses.
Results

cDNA sequencing detects FGFR2 amplification and a unique genomic profile in patients with FGFR2-amplified advanced GC

To evaluate the utility of cDNA compared to tissue samples for detecting FGFR2 amplification in advanced GC, we reviewed cDNA sequencing results of advanced GC from the GOZILA and GI-SCREEN studies as well as from publicly available tissue-based databases (The Cancer Genome Atlas [TCGA] and Memorial Sloan Kettering Cancer Center [MSKCC] databases). FGFR2 amplification was detected in 28 (7.7%) of 365 patients with advanced GC enrolled in the GOZILA study between January 2018 and January 2020 (Figure 1A). This prevalence was significantly higher than that detected by tissue sequencing in GI-SCREEN and publicly available tissue-based databases (GI-SCREEN: 3.4%, P = 0.00080; TCGA: 4.4%, P = 0.049; and MSKCC: 2.6%, P = 0.0027) (Figure 1A), which is consistent with a previous study reporting higher incidences of amplification of receptor tyrosine kinase genes in cDNA than tissue(33). Of 365 patients from GOZILA study, no cDNA alterations were detected in 54 (14.8%) patients. Very weak correlation between the FGFR2 plasma copy number (pCN) and cDNA maximum variant allelic frequency (max VAF) was observed (r² = 0.15, P = 0.041, Figure 1B). These findings indicated that cDNA sequencing may identify FGFR2 amplification that cannot be detected
by conventional tissue analysis.

Next, we evaluated if ctDNA could be used to identify other genomic features in FGFR2-amplified advanced GC. To this end, we compared concurrent genomic alterations between patients with FGFR2-amplified and nonamplified advanced GC. In FGFR2-amplified advanced GC, co-occurring amplifications of PIK3CA, MYC, CDK6, CCND1, BRAF, and CDK4 and mutations in ARID1A, BRCA2, and RHOA were detected at a significantly higher frequency than in GC without FGFR2 amplification (Figure 1C). The co-occurring amplification of ERBB2, MET, and EGFR was detected in 1 (3.6%), 3 (10.7%), and 6 (21.4%) of 28 patients with FGFR2 amplification, respectively. These suggest that ctDNA FGFR2-amplified advanced GC has a distinct profile of concurrent genomic alterations.

cDNA can detect FGFR2 amplification missed by tissue analyses

The increased prevalence of FGFR2 amplification in ctDNA suggests that ctDNA sequencing may detect heterogeneous FGFR2 amplification that tissue biopsy fails to identify. To assess whether ctDNA analysis can identify FGFR2 amplification missed by tissue analysis, we determined FGFR2 amplification in pretreatment tissue biopsy samples by IHC and FISH and in paired plasma samples obtained near the time of tissue biopsy
(median 2 days, interquartile range 1-4 days) by ctDNA sequencing in 44 patients with advanced GC (Figure 2A). No ctDNA genomic alteration was identified in four (9.1%) patients. FGFR2 amplification was detected by both tissue and ctDNA analysis in 6 patients and ctDNA analysis detected FGFR2 amplification in 6 additional patients, whereas no FGFR2 amplification was detected by tissue analysis only (Figure 2B). The pCN was not significantly different between tissue+ctDNA+ versus tissue−ctDNA+ (P = 0.18) (Figure 2B). No correlation of the CN detected in tissue and ctDNA analysis was observed in tissue+ctDNA+ and tissue−ctDNA+ patients (r² = 0.0025, P = 0.88, Figure 2C). Patients with FGFR2 amplification in ctDNA (tissue+ctDNA+ or tissue−ctDNA+) had a significantly shorter OS than those without FGFR2 amplification (tissue−ctDNA−) (median, 13.7 vs. 27.8 months; hazard ratio [HR] = 2.2; 95% confidence interval [CI], 1.0–4.9; P = 0.047) (Supplementary Figure 1). The OS of tissue−ctDNA+ patients was significantly shorter than that of tissue+ctDNA+ patients (median, 12.0 vs. 14.6 months; HR = 10.1; 95% CI, 1.1-90.8; P = 0.014) (Figure 2D). All of these patients received standard systemic chemotherapy, but not FGFR-targeted therapy. No statistically significant differences were observed in the clinicopathological characteristics among tissue+ctDNA+ and tissue−ctDNA+ patients (Supplementary Table 2). In addition, the median max VAF was 12.8 in the tissue+ctDNA+ and 10.6 in the tissue−ctDNA+ groups with no significant difference (P=0.52) (Supplementary
Figure 2). These findings suggest that ctDNA can identify FGFR2 amplification missed by tissue analyses, which is associated with a poorer prognosis.

Patients with **FGFR2 amplification identified by ctDNA analysis can benefit from FGFR inhibition therapy**

Previous studies had shown contradictory results regarding the efficacy of FGFR inhibitor therapy in patients with FGFR2-amplified advanced GC; since our results had shown that FGFR2 amplification might be missed by analysis of single-lesion tumor biopsies probably due to tumor heterogeneity, we next examined whether patients with FGFR2 amplification detected in ctDNA could achieve clinical benefit from an FGFR inhibitor. To this end, we highlight two patients who had FGFR2 amplification not detected by tissue analysis but identified by ctDNA sequencing and received an FGFR inhibitor.

Patient 1 was a 54-year-old man who had recurrent advanced GC with multiple lymph node metastases. NGS analysis of a tissue sample collected before primary tumor resection identified only a mutation in the *TP53* gene. This patient was treated with tegafur/gimeracil/oteracil (S-1) plus cisplatin as first-line, nanoparticle albumin-bound (nab)-paclitaxel plus ramucirumab as second-line, nivolumab as third-line, and irinotecan as fourth-line treatment. At the time of progression on nivolumab, ctDNA analysis using
Guardant360 in GOZILA identified FGFR2 amplification with a pCN of 24.2 as well as lower-level amplifications of ERBB2, CDK4, CCND1, and CCNE1, a subclonal FGFR2-TACC2 fusion, and mutations in NF1 and TP53 (Supplementary Table 3). Accordingly, following progression on irinotecan, the patient received an FGFR TKI based on the results of the ctDNA analysis. The patient achieved a -73.6% response with shrinkage of the metastatic cervical and axillary lymph nodes as target lesions (Figure 3A). The treatment was continued for three months, at which point disease progression occurred.

Patient 2 was a 57-year-old man who had unresectable GC with lymph node metastases and peritoneal dissemination. A pretreatment biopsy of the primary tumor showed only a PIK3CA mutation. This patient was treated with S-1 plus oxaliplatin as first-line, paclitaxel as second-line, and irinotecan as third-line treatment. The analysis of ctDNA using Guardant360 at the time of progression on irinotecan detected FGFR2 amplification with a pCN of 6.0 and mutations in APC and ARID1A (Supplementary Table 3). After progression on irinotecan, he received an FGFR TKI, which lead to the shrinkage of the thickened gastric wall and peritoneal dissemination with a decrease in carbohydrate antigen 19-9 (CA 19-9) (Figure 3B). To investigate changes in FGFR2 amplification patterns during chemotherapy, we retrospectively performed IHC and FISH analysis of tissue samples taken before treatment and after progression on S-1 plus oxaliplatin. Although
FGFR2 amplification was not detected in the pretreatment biopsy, the IHC and FISH analysis of the tissue sample obtained after S-1 plus oxaliplatin showed the emergence of high FGFR2 expression (score 4) and FGFR2 amplification with a CN of 32.9 (Supplementary Figure 3), suggesting that FGFR2 amplification emerged during the treatment or was missed by the initial single-site tissue biopsy in this case and was successfully identified by ctDNA-based sequencing.

These clinical responses to FGFR inhibitors suggest that patients with FGFR2 amplification identified by ctDNA sequencing but not detected by tissue analysis due to heterogeneity potentially benefit from treatment with FGFR inhibitors.

Concurrent MET amplification limited the clinical efficacy of FGFR inhibition therapy

Concurrent genomic alterations in FGFR2-amplified advanced GC shown in our study may be associated with the resistance to FGFR inhibition. We next report one patient with FGFR2 amplification and concurrent MET amplification in ctDNA who were treated with an FGFR inhibitor.

Patient 3 was a 64-year-old woman with unresectable GC with lymph node metastases and pleural and peritoneal dissemination. This patient was treated with 5-fluorouracil/leucovorin plus oxaliplatin as first-line and nab-paclitaxel plus ramucirumab as
second-line treatment. ctDNA sequencing using Guardant360 in GOZILA at the time of progression on nab-paclitaxel plus ramucirumab detected FGFR2 amplification with a pCN of 14.6 with concurrent MET amplification and mutations in TP53, CTNNB1, and ARID1A (Supplementary Table 3). On the basis of ctDNA analysis, she received an FGFR TKI. However, a CT scan at the first evaluation revealed not only progressive lymph node enlargement (+13.3%) but also the emergence of pleural effusion and a new brain metastasis within 30 days after the initiation of the investigational drug (Figure 4A, 4B). The tumor marker CA 19-9 increased from 476 to 937 U/mL during this period (Figure 4B). To identify potential genomic mechanisms of resistance, pre- and postprogression tissue and postprogression plasma were analyzed using OCA and Guardant360, respectively. Compared with the pretreatment pCNs, the pCN of FGFR2 amplification decreased after FGFR inhibitor therapy, falling from 14.6 to 7.3, while the pCN of MET amplification markedly increased from 3.0 to 15.7 (Figure 4C, Supplementary Table 3). Paired tissue NGS analysis also showed similar decreases in the FGFR2 CN and the emergence of MET amplification (Figure 4D). These dynamic changes in FGFR2 and MET amplification were confirmed by the IHC analysis of protein expression in the paired tissue samples (Figure 4E). Thus, in the patients with concurrent MET amplification, the clinical benefit of FGFR inhibitors may be limited by the outgrowth of MET-amplified clones that are not sensitive to
1 FGFR inhibition.

2
Discussion

The efficacy of FGFR inhibition has not been clear for FGFR2-amplified advanced GC with significant genomic heterogeneity, because more effective testing to detect FGFR2 amplification for this population has not been established before FGFR treatment. This study reveals that ctDNA sequencing can more frequently detect FGFR2 amplification than tissue analysis by identifying FGFR2 amplifications that may be missed by conventional tissue analysis. In addition, some patients with FGFR2 amplification identified only by ctDNA sequencing responded to treatment with an FGFR inhibitor. To our knowledge, this is the first report to show the efficacy of FGFR inhibition for advanced GC with amplified FGFR2 detected only by ctDNA sequencing.

Patients with FGFR2 amplification that is detected in ctDNA but undetectable by tissue analysis in our study might have FGFR2-amplified tumor cells in distant metastatic organs or primary tumors missed by single-lesion biopsy. These patients with tissue ctDNA+ for FGFR2 amplification tended to have poorer prognosis than those with FGFR2 amplification detectable in tissue, despite no statistically significant differences in the clinicopathological characteristics and max VAF among these groups. This is consistent with previous studies showing an association between genomic heterogeneity in GC and poor prognosis(34), although it remains possible that ctDNA shedding due to the tumor burden contributed to
this effect. These findings support that the utility of ctDNA sequencing in advanced GC for identifying genomic heterogeneity reported previously (22) can be applied for FGFR2-amplified disease.

In our case series, ctDNA detected FGFR2 amplification that was not detected with tissue testing, which may have occurred due to the acquisition of FGFR2 amplification in the interim between tissue and ctDNA testing and/or intratumoral heterogeneity that can be missed by single-lesion biopsies. Interestingly, the retrospective testing of a previously untested postprogression tissue sample in patient 2 confirmed the ctDNA finding, suggesting that FGFR2 amplification arose in this patient after initial chemotherapy or was missed by the initial single-lesion biopsy and successfully identified by ctDNA analysis, which integrates tumor cells throughout the body. This finding underscores the importance of genomic profiling of patients with advanced GC at each instance of disease progression, which can be achieved by ctDNA sequencing with minimal invasiveness in clinical practice.

In patient 3 in our study, ctDNA detected the concurrent amplification of FGFR2 and, to a lesser degree, MET. At primary progression on an FGFR inhibitor, the MET pCN was markedly elevated from baseline, while FGFR2 pCN was reduced, suggesting that two coexisting tumor cell subpopulations driven by different oncogenes responded to FGFR inhibition in opposing manners. Tumor tissue analysis and IHC confirmed that the dominant
FGFR2-expressing tumor cell population was replaced by MET-expressing clones upon progression. These findings strongly indicate the relevance of MET amplification in limiting the clinical efficacy of FGFR inhibition. This observation has particular relevance in light of our ctDNA genomic profiling, which revealed the frequent co-occurrence of FGFR2 amplification with other alterations, including ERBB2, EGFR, and MET amplifications. Others have similarly suggested that spatial intratumoral heterogeneity and concurrent genomic alterations in downstream molecules or other signaling pathways could act as resistance mechanisms to targeted therapies in advanced GC (23-26), leading to the frequent failure of targeted therapies. As such, ctDNA sequencing may also be useful for assessing concurrent genomic alterations to guide treatments.

An important caveat is that our findings were restricted to a Japanese population, although the frequency of tissue FGFR2 amplification in GI-SCREEN was consistent with that in the TCGA or MSKCC data sets. The analysis of paired tissue and plasma was conducted with a limited sample size due to the availability of plasma samples from the same timepoint as tissue collection. Furthermore, the frequency of FGFR2 amplification of this analysis was much higher than previously reported because this population included the patients previously known to have FGFR2 amplification. In addition, ctDNA genotyping potentially underestimated the frequency of FGFR2 amplification because some patients...
with gastric cancer have insufficient ctDNA shedding to detect genomic alterations in ctDNA,

although no patients in our study had FGFR2 amplification only in tissue, suggesting the

prevalence of FGFR2 amplification missed by ctDNA analysis due to the low amount of
tDNA may be limited. The efficacy of an FGFR inhibitor for FGFR2 amplification in ctDNA

was also shown only in two patients. The utility of ctDNA sequencing in detecting FGFR2

amplification in advanced GC needs to be investigated in prospective studies.

In conclusion, we report the utility of ctDNA sequencing for the detection of FGFR2

amplification that is missed by tissue analysis. Patients with such FGFR2 amplifications

have a poor prognosis when treated with standard nontargeted therapies but may benefit

from FGFR inhibitor treatment. We also found that concurrent MET amplification detected

by ctDNA sequencing was associated with limited the clinical efficacy of FGFR inhibition,
suggesting that combined FGFR and MET inhibition may be indicated in such cases. Taking

advantage of the ability of ctDNA sequencing to detect FGFR alterations, we are currently

conducting a phase II basket trial of futibatinib, an irreversible FGFR TKI, for patients with

solid tumors harboring FGFR alterations confirmed by Guardant360 (JapicCTI-194624)(35).

This trial will provide more validated evidence regarding the utility of ctDNA sequencing for

identifying FGFR alterations for FGFR-targeted therapy.
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Figure legends

Figure 1

Genomic characteristics of advanced gastric cancer (GC) with FGFR2 amplification based on ctDNA analysis. (A) Prevalence of FGFR2 amplification in advanced GC in GOZILA, GI-SCREEN, and the TCGA and MSKCC databases. (B) Correlations [Coefficient of determination ($r^2$)] between FGFR2 plasma copy number and maximum variant allelic frequency in FGFR2-amplified samples from GOZILA (n=28). (C) Prevalence of co-alterations in FGFR2-amplified (n = 28) versus nonamplified patients (n = 337) in GOZILA. Green and blue bars indicate prevalence of mutations and copy number variations co-altered with FGFR2 amplification, respectively. Prevalence in patients without FGFR2 amplification is highlighted in light colors. Abbreviations: ctDNA, circulating tumor DNA; TCGA, The Cancer Genome Atlas; MSKCC, Memorial Sloan Kettering Cancer Center; pCN, plasma copy number; max VAF, maximum variant allele frequency; mt, mutation; cnv, copy number variation.

Figure 2

Comparative analysis of paired tissue and plasma samples in advanced GC patients. (A) Schematic depicting analyses of paired synchronous primary tissue and plasma
samples in 44 advanced GC patients. (B) FGFR2 amplification status based on IHC (score), FISH (FGFR2/CEP10 ratio), and Guardant360 (pCN). Yellow boxes indicate high FGFR2 expression for tissue IHC score and FGFR2 amplification for tissue FISH or ctDNA sequencing. Low FGFR2 expression and no FGFR2 amplification are indicated by blue boxes. (C) Correlations [Coefficient of determination ($r^2$)] between FGFR2 pCN and tissue CN for patients with FGFR2 amplification detected in ctDNA. (D) OS based on the Kaplan–Meier method for patients with FGFR2 amplification detected in tissue*ctDNA* versus in tissue*ctDNA*. Abbreviations: GC, gastric cancer; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; pCN, plasma copy number; max VAF, maximum variant allele frequency; CN, copy number; ctDNA, circulating tumor DNA.

**Figure 3**

Treatment history and tumor evaluation by CT before treatment and best response in patients with FGFR2 amplification detected only by ctDNA who had tumor responses to FGFR inhibition with the shrinkage of target lesions (yellow allows). (A) Patient 1. (B) Patient 2. Abbreviations: CT, computed tomography; ctDNA, circulating tumor DNA.

**Figure 4**
Clinical presentation. (A) Tumor evaluation by CT at pretreatment and progression on an FGFR inhibitor with progressive lymph node enlargement and the emergence of a new brain metastasis (yellow arrow) in patient 3. (B) Changes in CA19-9 and sum of diameters of target lesions by CT following treatment with an FGFR inhibitor. (C) Change in the ctDNA pCN of FGFR2 and MET amplification before treatment and at progression on an FGFR inhibitor. (D) Change in the tissue CN of FGFR2 and MET amplification before treatment and at progression on FGFR inhibitor. (E) Hematoxylin and eosin stained and immunohistochemical stained images with anti-FGFR2 and MET antibodies of biopsy specimens of the primary gastric cancer before treatment and at progression on an FGFR inhibitor. Abbreviations: CT, computed tomography; CA19-9, carbohydrate antigen 19-9; ctDNA, circulating tumor DNA; pCN, plasma copy number; CN, copy number.
Figure 1
**Figure 2**

A. Diagram showing the process of tissue and plasma sample preparation.

B. Table showing the correlation between IHC and FISH scores.

C. Scatter plot showing the relationship between FGFR2 tissue CN and pCN.

D. Survival curve showing the probability of survival over time with FGFR2 amplification status.

Log rank P = 0.014
Hazard ratio for death = 10.1 (95% CI, 1.1-90.8)
Figure 3

A

TP53 G245S (NGS)

- 1st-line: S-1 + cisplatin
- 2nd-line: Nab-paclitaxel + ramucirumab
- 3rd-line: Nivolumab
- 4th-line: Irinotecan

FGFR2 amp

Tissue testing ctDNA sequencing

B

PIK3CA E542K (NGS)

FGFR2 negative (IHC/FISH) FGFR2 positive (IHC/FISH)

- 1st-line: S-1 + oxaliplatin
- 2nd-line: Paclitaxel
- 3rd-line: Irinotecan

FGFR2 amp

Tissue testing ctDNA sequencing

Pretreatment

Best response

5th-line: FGFR inhibitor

4th-line: FGFR inhibitor

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Figure 4
Circulating Tumor DNA Analysis Detects FGFR2 Amplification and Concurrent Genomic Alterations Associated with FGFR Inhibitor Efficacy in Advanced Gastric Cancer

Tomoko Jogo, Yoshiaki Nakamura, Kohei Shitara, et al.

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