Circulating tumor cells with FGFR2 expression might be useful to identify patients with existing FGFR2-overexpressing tumor

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Abstract
Fibroblast growth factor receptor (FGFR) is associated with proliferation, migration, and angiogenesis of carcinomas, and FGFR signaling inhibitors are considered a key drug for the treatment of solid tumors with FGFR overexpression. Amplification of FGFR2 is reportedly identified in 3%-10% of gastric cancers (GCs). The aim of this study is to clarify whether the identification of the circulating tumor cells (CTCs) with FGFR2 overexpression is useful to detect patients with FGFR2-overexpressing GC. One hundred GC patients who underwent gastrectomy were enrolled. A total volume of 8 mL of peripheral blood was collected from each patient just before gastrectomy, and mononuclear cells were enriched by Ficol density gradient centrifugation. These cells were immunostained with PI/CD45/EpCAM/FGFR2. The number of CTCs with FGFR2 expression in each sample was enumerated by FACScan. The FGFR2 expression level of the resected primary tumor was assessed by immunohistochemistry. The number of FGFR2-positive CTCs in the GC patients' peripheral blood was significantly correlated with the FGFR2 expression level of the primary GC. The relapse-free survival of the patients with FGFR2-positive CTCs (≥5 cells/10 mL blood) was significantly poorer (P = .018, log-rank) than that of the patients without FGFR2-positive CTCs (<5 cell/10 mL blood). These findings suggested that the determination of FGFR2-positive CTCs might help identify an existing tumor with FGFR2 overexpression.

KEYWORDS
clinical study, CTC, FGFR2, flow cytometry, gastric cancer

1 | INTRODUCTION

Despite recent advances in diagnostic devices and therapies for gastric cancer (GC), the prognosis of this cancer remains poor; GC is the fifth most common lethal carcinoma and the third leading morbidity in the world.¹-³ The clinical use of targeted therapy for specific molecules involved in the genesis and progression of cancer has been increasing and has improved the prognosis of many cancer patients.⁴-⁶

Fibroblast growth factor receptor (FGFR), a transmembrane receptor tyrosine kinase, has several roles in the genesis of cancer.⁷,⁸

Abbreviations: APC, allophycocyanin; CD45, cluster of differentiation 45; CK, cytokeratin; CTC, circulating tumor cell; DMEM, Dulbecco's Modified Eagle Medium; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; FGFR, fibroblast growth factor receptor; FITC, fluorescein isothiocyanate; GC, gastric cancer; PBS-T, phosphate buffered saline with Tween 20; PI, propidium iodide.
FGFR2 amplification is reportedly identified in 3%-10% of primary GCs.8,10 FGFR signaling inhibitors have been considered a key drug in the treatment of GCs that show FGFR2 overexpression.11 A number of FGFR inhibitors such as AZD4547,12 NVP-BGJ398,13 TAS120,14 E7090,15 LY287445,16 BAY1163877,17 and bemarituzumab (FPA144) were developed in recent years. A phase I study of FPA144 showed a good disease control rate for gastroesophageal cancer patients with FGFR2-IIIb overexpression,18 and a phase III trial has been ongoing for FGFR2-positive GC patients (NCT03343301).3 However, a phase II study of AZD4547 was performed, and AZD4547 did not improve the prognosis of GC patients with FGFR2 amplification (NCT01457846).12 One of the reasons for the negative results of the clinical study might be the tumor heterogeneity between primary and recurrent tumors.

Most of the prior molecular analyses for the determination of target molecules used surgical resected specimens or biopsy specimens from patients, and the specimens were examined by immunohistochemistry, fluorescence in situ hybridization (FISH), or polymerase chain reaction (PCR).4,19 However, differences in the driver molecule and molecular characterization of inaccessible tumors with tumor heterogeneity that will benefit from targeted therapy.25-29 This suggests that the assessment of CTCs might overcome the issue of heterogeneity among cancer patients. The present study was conducted to clarify whether FGFR2 expression on CTCs would provide an accurate indication of the FGFR2 status of existing tumors in GC patients. Also, we assessed the correlation between CTCs with FGFR2 expression and the prognosis of GC patients. To the best of our knowledge, this is the first study to investigate the clinical significance of FGFR2+ CTCs in the peripheral blood of GC patients.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Human GC cell lines, OCUM-2MD3,30 KATO-III, MKN45, and NUGC4 (JCRB Cell Bank) were used. The cells were cultured with DMEM (Wako) at 37°C in 21% O2 with 5% CO2.

2.2 | Western blot analysis

Cell lysates were collected after different treatments. Each sample was subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was placed in PBS-T solution containing each primary antibody: FGFR2 (1:200; Cell-Signaling), pFGFR (Y653/654) (1:200; Cell-Signaling), or β-actin (1:1000; Sigma-Aldrich) at 4°C for overnight. The blots were incubated with secondary antibody for 30 minutes and were detected by enhanced chemiluminescence using ECL prime (GE Health Care).

2.3 | Spike test

A total volume of 8 mL of peripheral blood was drawn from a healthy volunteer. A total of 250 cultured tumor cells of each four GC cell lines was spiked into 2 mL of the healthy volunteer’s peripheral blood. Peripheral blood mononuclear cell fraction was enriched by Ficol at 1500 g for 15 minutes, followed by dividing into four samples. The sample was stained by propidium iodide (PI; BD Bioscience), anti-human CD45 (BD Bioscience), anti-FGFR2 (mFR2, Daiichi Sankyo Co) conjugated with Alexa Fluor 488 (G100L-Alexa 488), and anti-EpCAM conjugated with APC (BD Bioscience: 347200). As control, the sample was stained with IgG1 isotype control. Flow cytometry analysis was performed on a FACScan (BD LSR II; Becton Dickinson). When the cutoff value of FGFR2-positive fluorescence was determined 1000, the FGFR2-positive number of OCUM-2MD3 and KATO-III was high, and the FGFR2-positive number of MKN45 and NUGC3 was low in the spike test. FGFR2+ cells were determined FGFR2 Alexa488 >103 after the exclusion of PI-positive and CD45-positive cells. Isotype negative control was defined as PI-negative and CD45-negative to exclude nonspecific binding of the primary antibody.

2.4 | Sample preparation from GC patients’ peripheral blood for CTC analysis

One hundred patients with GC who underwent gastrectomy at Osaka City University Hospital between December 2016 and January 2019 were enrolled. The pathological diagnoses and classifications were made according to the Japanese Classification of Gastric Carcinoma (14th edition).31 This study was approved by the ethics committee of Osaka City University Hospital.

FIGURE 1  Fibroblast growth factor receptor2 (FGFR2) expression and determination of gastric cancer cells in peripheral blood. A, Western blot analysis of FGFR2/phospho-FGFR (pFGFR) expression. KATO-III cells and OCUM-2MD3 cells expressed both FGFR2 and pFGFR2, but MKN45 cells and NUGC3 cells did not. B, Determination of FGFR2+ cells in peripheral blood of a healthy volunteer by flowcytometry. No FGFR2+ cell was detected when the cutoff value of FGFR2+ fluorescence was 1000. C, Detection of FGFR2+ gastric cancer cells in the peripheral blood of a healthy volunteer by flowcytometry. FGFR2+ cells of OCUM-2MD3, KATO-III, MKN45, and NUGC3 were detected (101 cells, 177 cells, 4 cells, and 9 cells of a total of 250 cells, respectively). D, H&E staining of FGFR2+ cells and cultured KATO-III cells. The morphology of FGFR2+ cells sorted by FACScan is similar to that of cultured KATO-III cells. E, FGFR2 immunostaining and FGFR2 FISH of cancer cell lines sorted by FACScan. FGFR2 expression and FGFR2 amplification were found in OCUM-2MD3- or KATO-III-spiked peripheral blood but not in MKN45- or NUGC-spiked peripheral blood.
(A) pFGFR, FGFR2, β-actin

(B) FGFR2 (Axera Flour 488)

(C) OCUM-2MD3

D) FGFR2+ cells sorted by FACS

(KATO-III cells)

(E) H.E., FGFR2 IHC, FGFR2-FISH
by the Osaka City University Ethics Committee (approval number 3159). Informed consent was obtained from all patients, and the study has been conducted according to the principles of the Declaration of Helsinki. A total volume of 8 mL of peripheral blood was collected from GC patients prior to surgery. Peripheral blood mononuclear cells were enriched by Ficol. The samples were immunostained with PI, CD45, EpCAM, FGFR2 (G100L-Alexa 488), IgG1 isotype control, and CK, as described above.

2.5 | Immunohistochemical determination of FGFR2

FGFR2 expression of resected specimens was assessed by immunohistochemical staining using the anti-human FGFR2 (mFR2) for 60 minutes at room temperature. The percentage of FGFR2-expressing cells at membrane or cytoplasm was evaluated, as previously reported by AL Paterson et al.\textsuperscript{32} FGFR2 expression was categorized into four groups as follows: 0, no staining; 1+, cytoplasmic staining in 1%-80% or membranous staining in 1%-5%; 2+, cytoplasmic staining in 80%-100% or membranous staining in 5%-19%; 3+, membranous staining in 20%-100%. The evaluation was done by two double-blinded distinct observers without being aware of clinical data and outcome.

2.6 | FISH of FGFR2

Randomly selected 28 cases were examined for FGFR2 gene amplification with FISH. The immunohistochemistry-positive area of tumor paraffin-embedded sections were subjected to FISH. Tumor sections were deparaffinized with the pretreatment reagent (Abbott Molecular Inc). After protease digestion procedures, FGFR2/CEN10p dual color FISH probes were hybridized at 75°C for 5 minutes and 37°C for 48 hours. FGFR2/CEN10p, both of which are located on chromosome 10, was defined as the ratio of the number of FGFR2 signals to the number of CEN10p signals, and FGFR2/CEN10p ≥2 was adopted as FGFR2 amplification. In each case, FGFR2 and CEN10p signals of 40 tumor cell nuclei were counted, and the average of FGFR2/CEN10p was obtained.

2.7 | Statistical analysis

We used the chi-squared test or Fisher’s exact test to determine the significance of the difference between the covariates. A Kruskal-Wallis test with post-hoc comparison was used to compare differences between two independent groups. The survival durations were calculated using the Kaplan-Meier method and analyzed by the log-rank test. The Cox proportional hazards model was used to compute multivariate hazards ratios for the study parameters. In all of the tests, a P value <.05 was defined as statistically significant. Statistical analysis was performed using R for Mac OS X (version 3.5.2).

3 | RESULTS

3.1 | Determination of GC cells with FGFR2 expression in peripheral blood by spike test

The Western blot analysis revealed that KATO-III cells and OCUM-2MD3 cells expressed phosphorylated FGFR2, while MKN45 cells and NUGC4 cells did not (Figure 1A). When the cutoff value of FGFR2 fluorescence was determined 1000 by IgG1 isotype control. FGFR2 cells were not detected in the healthy volunteer’s peripheral blood (Figure 1B). On the other hand, a total of 101 (40%) FGFR2 cells were present in OCUM-2MD3–spiked peripheral blood, and 177 (71%) cells were present in KATO-III–spiked peripheral blood. In contrast, only four (2%) and nine (4%) FGFR2 cells were detected in MKN45-spiked and NUGC3–spiked peripheral blood, respectively (Figure 1C). The morphology of the FGFR2 cells sorted by FACS in KATO-III–spiked peripheral blood was similar to that of the cultured KATO-III cells (Figure 1D), which suggested that FACS analysis might detect cancer cells with FGFR2 overexpression in peripheral blood. Expression of FGFR2 and FGFR2 gene amplification were found in OCUM-2MD3
Abbreviation: FGFR2

+ detected in peripheral blood by FACS, and two cells were EpCAM + cells were of a GC patient by FACS analysis. A total of eight FGFR2 Figure 2 shows the representative scattered graph of peripheral blood GC patients

3.2 FACS analysis of peripheral blood of GC patients

Figure 2 shows the representative scattered graph of peripheral blood of a GC patient by FACS analysis. A total of eight FGFR2 + cells were detected in peripheral blood by FACS, and two cells were EpCAM + CK in this case. In the 100 GC cases, FGFR2 + cells were detected in 50 (50%) cases (Table 1). Among these 50 cases with FGFR2 +, EpCAM + cells were detected only in two cases (data not shown).

3.3 The correlation between FGFR2 + CTCs and the FGFR2 immunostaining in GC

Figure 3A shows representative FGFR2 immunostaining of primary gastric tumors which were taken by gastrectomy. FGFR2 immunostaining indicated that cancer cells overexpressed FGFR2 at either the cell membrane and/or the cytoplasm. The number of IHC-0 cases, IHC-1 + cases, IHC-2 + cases, and IHC-3 + cases was 39, 35, 17, and 9, respectively. The number of FGFR2 + CTCs in the 2 mL of blood from GC patients was 0.6 ± 1.2, 2.4 ± 4.2, 2.6 ± 2.9, and 8.3 ± 11.2 (mean ± SD) in the IHC-0, IHC-1 +, IHC-2 +, and IHC-3 + groups, respectively. The number of FGFR2 + CTCs increased in accordance with the FGFR2 IHC level. A significant difference was found between the IHC-3 + group and the IHC-0 group (P < .01), IHC-1 + group (P < .01), and IHC-2 + group (P < .01) by the Mann-Whitney U-test (Figure 3B).

3.4 The correlation between FGFR2 + CTCs and FGFR2 FISH in GC

Figure 4A provides representative pictures of the FGFR2 FISH analysis. The FGFR2/CEN10 ratio in the IHC-0, IHC-1 +, IHC-2 +, and IHC-3 + groups was 1.1 ± 0.1, 1.2 ± 0.2, 1.2 ± 0.3, and 2.1 ± 0.3 (mean ± SD), respectively. The FGFR2/CEN10 ratio significantly increased in accordance with the FGFR2 IHC level. (Figure 4B). There was significantly positive correlation between the number of FGFR2 + CTCs and the FGFR2/CEN10 ratio (r = .515; Spearman’s rank correlation coefficient, P = .002; Figure 4C).

3.5 The correlation between FGFR2 + CTCs and CK + EpCAM + CTCs

The number of CK + EpCAM + cells of the T3 and T4 cases was significantly greater (P < .001) than that of the T1 and T2 cases. The number of CK + EpCAM + cells of the lymph node metastasis cases was significantly larger (P = .014) than that of the no–lymph node metastasis cases (Figure 5A). Spearman’s rank correlation test indicated that FGFR2 + CTCs were not correlated with CK + EpCAM + CTCs (r = -.189, P = .060) (Figure 5B).

3.6 The relationship between the FGFR2 + CTCs and clinicopathological features

The clinicopathological characteristics of all 100 patients according to their FGFR2 + CTC values are summarized in Table 1. The cutoff value

| Table 1 | Relationship between the FGFR2+ circulating tumor cells (CTCs) and clinicopathologic features in 100 gastric cancer cases |
|---|---|---|
| | Number of FGFR2+ cells | |
| | ≥5 cells/10 mL (n = 50) | <5 cells/10 mL (n = 50) | P value |
| Age | | | |
| ≤70 | 21 (42.0%) | 31 (62.0%) | .071 |
| >70 | 29 (58.0%) | 19 (38.0%) | |
| Gender | | | |
| Female | 20 (40.0%) | 18 (36.0%) | .837 |
| Male | 30 (60.0%) | 32 (64.0%) | |
| Macroscopic type | | | |
| Borrmann’s type 4 | 0 (0.0%) | 6 (12.0%) | .15 |
| Other types | 50 (100%) | 44 (88.0%) | |
| Microscopic type | | | |
| Differentiated | 27 (54.0%) | 27 (54.0%) | 1.00 |
| Undifferentiated | 23 (46.0%) | 23 (46.0%) | |
| Depth of invasion | | | |
| T1/ T2 | 26 (52.0%) | 31 (62.0%) | .419 |
| T3/ T4 | 24 (48.0%) | 19 (38.0%) | |
| Lymph node metastasis | | | |
| N0 | 37 (74.0%) | 37 (74.0%) | 1.00 |
| N1/ N2/ N3 | 13 (26.0%) | 13 (26.0%) | |
| Lymphatic invasion | | | |
| Negative | 27 (54.0%) | 31 (62.0%) | .544 |
| Positive | 23 (46.0%) | 19 (38.0%) | |
| Venous invasion | | | |
| Negative | 32 (64.0%) | 37 (74.0%) | .387 |
| Positive | 18 (36.0%) | 13 (26.0%) | |
| Tumor size [median] | 25.7 [2.0-40.0] | 20.0 [1.0-33.5] | .525 |
| Stage | | | |
| I and II | 36 (72.0%) | 36 (72.0%) | 1.000 |
| III and IV | 14 (28.0%) | 14 (28.0%) | |
| CEA [median] | 1.4 [0.5-3.7] | 1.6 [0.5-2.6] | .377 |
| CA19-9 [median] | 1.0 [1.0-22.0] | 1.0 [1.0-11.8] | .232 |

Abbreviation: FGFR2+, fibroblast growth factor receptor 2+.
for the number of FGFR2+ CTCs was determined as one cell per 2 mL peripheral blood based on the results of the receiver-operating characteristics curve for relapse-free survival (RFS) as the endpoint. Of all 100 patients enrolled in this study, the number of patients with one or more FGFR2-positive CTCs was 50. There was no significant difference between the FGFR2+ CTC group and the FGFR2- CTC group.
Survival was analyzed for 89 patients (excluding the 11 pStage IV patients from the total of 100 patients). Among the 89 patients, recurrence-free survival of patients with FGFR2\(^+\) cells \(\geq\) one cell per 2 mL was significantly poorer \((P = .018, \text{log-rank test})\) than that of patients with no FGFR2\(^+\) cells (Figure 6A). On the other hand, there was no significant difference of RFS between patients with CK\(^+\) EpCAM\(^+\) and those without CK\(^+\) EpCAM\(^+\) (Figure 6B). The number of recurrence patients was significantly greater (Fisher, \(P = .026\)) in FGFR2-positive CTCs cases (11 of 47 patients) than in FGFR2-negative CTCs cases (3 of 42 patients). Table 2 summarizes the results of the univariate and multivariate analyses for RFS. The univariate analysis revealed that poor survival was significantly correlated with present FGFR2\(^+\) CTCs \(\geq\) 5/10 mL \((P = .029)\), macroscopic type 4 \((P < .001)\), microscopic undifferentiated type \((P = .017)\), high T stage \((P = .003)\), and lymph node metastasis \((P = .002)\). The multivariate analysis revealed that present FGFR2\(^+\) cells \((P = .042)\), macroscopic type 4 \((P = .003)\), and lymph node metastasis \((P = .020)\) were independent prognostic factors.

### 4 | DISCUSSION

The present results demonstrated that the existence of FGFR2\(^+\) CTCs in the peripheral blood of GC patients was significantly correlated...
with FGFR2 overexpression and FGFR2 gene amplification in gastric tumor. These findings suggest that the analysis of FGFR2+ CTCs is useful to identify patients who have an existing recurrent tumor with FGFR2 overexpression.

Several clinical trials using FGFR2-selective inhibitors have been conducted. Some studies obtained positive results, or are ongoing, while other studies obtained negative results. One of the reasons for the negative results of clinical studies of FGFR2 inhibitors might be the tumor heterogeneity of FGFR2 expression between the resected specimen and the existing tumor. FGFR2+ CTCs might help detect the existing FGFR2-overexpressing tumor. Our present findings indicate that FGFR2+ CTCs might be a promising predictive biomarker to overcome the issue of tumor heterogeneity of GC patients with FGFR2-overexpressing tumors.

The determination of CK+EpCAM+ cells is a standard method for identifying CTCs in several types of cancer. Although CK+EpCAM+ CTCs were detectable in peripheral blood, CK+EpCAM+ CTC was not associated with poor prognosis in this study. It has been reported that CK-positive or EpCAM-positive CTCs were not correlated with the prognosis of patients with a solid tumor including GC. These findings suggest that CK+EpCAM+ CTCs might have high malignant potential for GC patients. On the other hand, our results indicated that the prognosis of patients with FGFR2+ CTCs was significantly poorer than that of patients without FGFR2+ CTCs. There are two isoforms of the FGFR2: an FGFR2-IIIb isotype, which is expressed mainly in epithelial cells, and an FGFR2-IIIc isotype, which is expressed mainly in mesenchymal cells. The switch from FGFR2-IIIb to FGFR2-IIIc by selective splicing is involved in the epithelial-mesenchymal transition (EMT), an important process of metastasis and invasion. In the present investigation, most of the FGFR2+ CTCs showed low EpCAM expression. Our FGFR2 antibody recognizes not only the FGFR2-IIIb isotype but also the FGFR2-IIIc isotype of cancer cells exhibiting the EMT. Most FGFR2+ CTCs might be EMT cancer cells with FGFR2-IIIc expression, which might be one of the reasons for the finding that the number of FGFR2+ cells was not correlated with that of CK+EpCAM+ cells, and FGFR2+ CTCs can be used as a prognostic factor.

One of the limitations of this study is that the clustered CTCs were not evaluated because doublet cells were excluded in the FACS analysis. As it has been reported that the cluster formation of CTCs might promote metastasis, in future it would be necessary to analyze the clinical significance of single CTCs versus CTC clusters. Another limitation is that postoperative CTCs were not evaluated in this study. The postoperative monitoring of FGFR2+ CTCs might be necessary to clarify whether FGFR2+ CTCs are useful to predict high-risk patients for recurrence and to determine patients for the indication of FGFR2 inhibitors.

In conclusion, the determination of FGFR2+ CTCs might be useful to identify GC patients with existing tumor with FGFR2 overexpression. The presence of FGFR2+ CTCs might be a promising prognostic factor in GC patients.

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### DISCLOSURE
Masakazu Yashiro received research funding from Daiichi Sankyo Co., Ltd.

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**TABLE 2** Univariate and multivariate analysis for relapse-free survival

| Variables                        | Univariate analysis | Multivariate analysis |
|----------------------------------|---------------------|-----------------------|
|                                  | Hazard ratio (95% CI) | p value | Hazard ratio (95% CI) | p value |
| FGFR2+ cell ≥5 cells/10 mL       | 4.07 (1.16-14.3)    | .029     | 4.87 (1.06-22.4)     | .042    |
| Age > 70 y-old                   | 1.17 (0.44-3.13)    | .750     |                      |         |
| Gender                           |                     |          |                      |         |
| Female vs Male                   | 1.20 (0.45-3.22)    | .719     |                      |         |
| Macroscopic type                 |                     |          |                      |         |
| Borrmann’s type 4                | 13.1 (1.57-108.8)   | .017     | 69.3 (4.13-1164)     | .003    |
| Microscopic type                 |                     |          |                      |         |
| Undifferentiated type            | 1.22 (0.45-3.27)    | .695     |                      |         |
| Depth of invasion T3/4 vs T1/2   | 5.09 (1.76-14.7)    | .003     | 1.86 (0.55-6.29)     | .319    |
| Lymph node metastasis            |                     |          |                      |         |
| N1/2/3 vs N0                     | 5.00 (1.74-14.4)    | .002     | 4.51 (1.26-16.0)     | .020    |

Abbreviation: CI, confidence interval; FGFR2+, fibroblast growth factor receptor2+.
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