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Genome-wide and size-based cell-free DNA indices as predictive biomarkers for locally advanced esophageal squamous cell carcinoma treated with preoperative or definitive chemoradiotherapy

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ABSTRACT

For locally advanced esophageal cancer, concurrent chemoradiotherapy (CRT) followed by surgery has been a standard treatment, while clinical studies showed comparable survival outcomes between definitive CRT and neoadjuvant CRT followed by surgery in patients responding to CRT. Thus, biomarkers are required to predict treatment outcomes and benefit of adding surgery after CRT. This prospective biomarker study examined the role of cell-free DNA (cfDNA) fragmentation profiles and genomic copy number variations (CNVs) in predicting treatment outcomes in esophageal squamous cell carcinoma patients treated with neoadjuvant or definitive CRT. The clinical response was evaluated after induction chemotherapy and after CRT. Fragment Ratio (FR)-score and I-score were calculated from plasma cfDNA reflecting fragment

Abbreviations: CRT, concurrent chemoradiotherapy; cfDNA, cell-free DNA; CNVs, copy number variations; FR-score, Fragment Ratio; ctDNA, circulating tumor DNA; ESCC, esophageal squamous cell carcinoma.

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lengths and CNV of cfDNA, respectively. The association between indices of cfDNA (cfDNA concentration, FR-score, and I-score) and treatment outcomes (clinical response, time to progression [TTP], and overall survival [OS]) were evaluated. Sixty-one patients were included. Thirty patients received neoadjuvant CRT followed by surgery, whereas 31 received definitive CRT. Low baseline, post-induction chemotherapy, and post-CRT FR-scores and low post-induction I-score were significantly associated with improved treatment response ($P < 0.05$). Additionally, patients with surgery after CRT showed significantly longer survival than patients without surgery in the FR-score-high group (median TTP, 12.7 vs 3.4 months; $P = 0.011$; OS, not reached vs 12.9 months; $P = 0.02$), while there was no survival benefit with surgery in the FR-score-low group. FR-score may be a new biomarker to predict treatment response, residual tumor burden after CRT, and consequently, survival benefit of adding morbid surgery after CRT. FR-score has strength in a relatively simple and inexpensive methodology compared to deep sequencing, resulting in high availability and accessibility, despite limited sensitivity.

ARTICLE INFO

Keywords: Cell-free DNA, Circulating tumor DNA, FR-score; Esophageal cancer, Biomarker, Concurrent chemoradiotherapy

Introduction

Esophageal cancer is one of the most deadly malignancies, which is the seventh most common cancer and the sixth most common cause of cancer-related death in the world.[1] Surgical resection remains the standard of treatment for localized resectable esophageal cancer. However, in locally advanced disease, surgery alone usually results in an unsatisfactory prognosis. Hence, the addition of neoadjuvant concurrent chemoradiotherapy (CRT) has been established as standard therapy by demonstrating survival benefits in several phase III studies.[2,3] Definitive CRT is treatment of choice for patients who has locally advanced unresectable esophageal cancer, cannot tolerate or decline surgery. Clinical studies have shown comparable survival outcomes between definitive CRT and neoadjuvant CRT followed by surgery, particularly in patients who responded to CRT.[4,5] Although these multidisciplinary approaches improved treatment outcomes in esophageal cancer, there remains an urgent need to develop better biomarker-guided treatment strategies.

Recently, circulating tumor DNA (ctDNA) has gained increasing attention as a noninvasive biomarker in various cancer types. Blood ctDNA represents only 0.01%-10% of total circulating cell-free DNA (cfDNA), most of which originates from normal cells of the body.[6] Previous studies have shown the potential of ctDNA using its concentration or genomic alterations in various clinical settings including diagnosis, detection of residual disease, selection of targeted therapy, monitoring treatment response and resistance.[7] Interestingly, recent studies have reported that ctDNA varies in size and is shorter than normal cfDNA. Furthermore, mutant alleles of ctDNA more commonly have shorter fragment lengths.[8-11] Cristiano et al[11] suggested that fragmentation patterns of cfDNA across the genome could be used the screening, early detection, and monitoring of human cancer. However, few studies have investigated the prognostic and predictive impact of fragmentation profiles of cfDNA in esophageal cancer patients.

Thus, the aim of this study was to examine the role of cfDNA fragmentation profiles and genomic copy number variations (CNVs) across the whole genome to predict treatment outcomes in patients with esophageal squamous cell carcinoma (ESCC) treated with neoadjuvant or definitive CRT.

Methods

Study population

This prospective biomarker study was conducted under approval from Institutional Review Board at Asan Medical Center, Republic of Korea. Patients were included based on the following criteria: (1) age $\geq$18 years; (2) histologically confirmed ESCC; (3) locoregional disease according to the eighth edition of American Joint Committee on Cancer staging system. However, patients with M1 disease due to supraclavicular lymph node metastasis were included; (4) treated with neoadjuvant or definitive CRT, and (5) available peripheral blood samples. Exclusion criteria were as follows: (1) prior treatment for esophageal cancer except surgery; (2) concurrent other malignancy that might affect clinical outcomes; and (3) no available clinical data for response evaluation to CRT. All patients provided written informed consent before enrollment.

Plasma samples from 97 healthy adult volunteers were used as negative controls and were collected after obtaining informed consent.

Treatment and assessment

CRT consisted of capecitabine plus cisplatin or paclitaxel, carboplatin plus pembrolizumab (as part of a phase II trial of preoperative CRT with pembrolizumab for ESCC, NCT02844075) concurrently administered with radiotherapy. Radiotherapy was delivered to a total dose of 40-54 Gy in 21-28 fractions. Before CRT, 2 cycles of induction chemotherapy were administered with the same regimen as CRT according to the institutional practice, although this was omitted in 7 patients included in the clinical trial described above.

Pretreatment staging work-up is described in Supplementary Methods. After induction chemotherapy, metabolic response using ^{18}F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) was assessed as described in Supplementary Methods.[12] Approximately 4-8 weeks after the end of CRT, overall clinical response was re-evaluated with endoscopy with biopsy, chest computed tomography (CT), and PET, and was classified as described in Supplementary Methods.[13]

Patients with resectable disease who were medically fit underwent surgical resection approximately 4-8 weeks after the end of CRT. While patients showing progressive disease (PD) to CRT received subsequent treatment at the physician's discretion, those who achieved complete response (CR), partial response (PR), or stable disease (SD) were followed up without treatment until disease progression. Time to progression (TTP) and overall survival (OS) were assessed from the time of the response evaluation after CRT.

Blood sample collection and cfDNA extraction

Peripheral blood samples were collected at baseline, after induction chemotherapy, and at the time of response evaluation after CRT. Samples were centrifuged to isolate the plasma, which were then stored at –80°C until cfDNA extraction. cfDNA was extracted from each patient using the Tiangen micro DNA kit (Tiangen Biotech, Beijing, China). Final DNA eluent (50 μL) was quantified by Qubit 2.0 Fluorometer with Qubit dsDNA HS (High Sensitivity) assay kit (Life Technology, Carlsbad, CA).

Library preparation for whole-genome sequencing

DNA library preparation was performed as described in Supplementary Methods.

Data analysis for calculation of genome instability and fragmentation

Whole-genome sequencing (WGS) data were analyzed as described in Supplementary Methods.[14-16] To enrich the ctDNA proportion among cfDNA, in silico size selection (specified size range: 90-150 bp) of the sequencing reads was performed using samtools (v1.7).[16,17] Then, we divided the entire autosomal genome into nonoverlapping 100 Kb bins, and the coverage of each bin was calculated. GC correction was performed using the LOESS algorithm. The Z-score for each bin was calculated using the mean and standard deviation (SD) of 20 healthy control subjects and smoothing was performed using the LOESS algorithm.

$$Zscore_{bin} = \frac{Normalized\ percentage\ of\ read\ count\ in\ the\ bin_{sample} - Mean\ normalized\ percentage\ of\ read\ count\ in\ the\ bin_{control}}{SD\ of\ normalized\ percentage\ of\ read\ count_{control}}$$

To express whole genome instability, we used I-score which was calculated as follows[18]:

$$I\text{-}score = \log 2\left(\sum_{i=autosome\ bins} |LOESS\ smoothed\ Z_i|\right)$$

Higher I-score indicates higher chromosomal instability.

We also developed Fragment Ratio (FR)-score which reflects the ratio of short and long fragments of cfDNA. After mapping quality filtering, 40 million reads were randomly extracted from all reads to normalize all sample's sequencing depth variation. Then fragments were divided into a short ($90 < length < 150$) and long fragment group ($151 < length < 210$), and their ratio was calculated for each 1 Mb nonoverlapping bins. Then, this value was converted into a Z-score and the LOESS algorithm was used to perform GC-correction. FR-score was calculated as follows:

$$FR - score = \log 2\left(\sum\nolimits_{i=autosome\ bins} LOESS\ smoothed\ GC\ normalized\ FRZ_i^{\,2}\right)$$

Higher FR-score indicates higher proportion of short fragments in cfDNA, which are associated with higher proportion of ctDNA fragments in cfDNA.[8-11]

Statistical analysis

Mann-Whitney U and Kruskal-Wallis tests for independent samples and Wilcoxon signed-rank and Friedman tests for dependent samples were used for continuous variables. Chi-square or Fisher's exact tests were used for categorical variables. Pearson's correlation co-efficient was calculated to determine the correlation between cfDNA indices. Kaplan-Meier method was used to estimate the distributions of time to event outcomes, and log-rank test was used to test difference between groups. All tests were 2-sided, and P values < 0.05 were considered statistically significant.

Results

Patient characteristics

Among 63 patients who were enrolled in the current study between March 2017 and December 2018, 2 patients who did not receive CRT because of esophageal perforation during induction chemotherapy or patient's refusal, were excluded, leaving 61 eligible for the analysis. Baseline characteristics are described in Table 1. Thirty patients received neoadjuvant CRT followed by surgery, while 31 received definitive CRT.

Table 1
Baseline characteristics.

Characteristics		No. of patients (N = 61)
Age, years (range)	Median	61 (41-76)
Sex	Male	55 (90.2%)
	Female	6 (9.8%)
ECOG performance status	0	8 (13.1%)
	1	52 (85.2%)
	2	1 (1.6%)
Tumor location	Cervical (UI 15-20 cm)	2 (3.3%)
	Upper thoracic (UI 20-25 cm)	11 (18.0%)
	Mid thoracic (UI 25-30 cm)	15 (24.6%)
	Lower thoracic (UI 30-40 cm)	33 (54.1%)
Histologic grade	G1 (W/D)	9 (14.8%)
	G2 (M/D)	40 (65.6%)
	G3 (P/D)	6 (9.8%)
	GX (not assessed)	6 (9.8%)
Clinical T stage*	T0†	3 (4.9%)
	T1	10 (16.4%)
	T2	7 (11.5%)
	T3	31 (50.8%)
	T4	10 (16.4%)
Clinical N stage*	N0	13 (21.3%)
	N1	31 (50.8%)
	N2	16 (26.2%)
	N3	1 (1.6%)
Clinical TNM stage*	I	9 (14.8%)
	II	12 (19.7%)
	III	20 (32.8%)
	IVA	4 (6.6%)
	IVB (SCN metastasis only as M1)	16 (26.2%)
Disease status	De novo	58 (95.1%)
	Recurrent	3 (4.9%)
Induction chemotherapy	Done	54 (88.5%)
	Not done	7 (11.5%)
Chemotherapy regimen for CRT	Capecitabine and cisplatin	54 (88.5%)
	Paclitaxel, carboplatin and pembrolizumab	7 (11.5%)
Total radiation dose, Gy	Median	50 (28-54)
Chemoradiotherapy	Preoperative	30 (49.2%)
	Definitive	31 (50.8%)

ECOG PS, Eastern Cooperative Oncology Group performance status; UI, upper incision; W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; SCN, supraclavicular node.
Data are median (range) or number (%).
* Clinical staging was done according to the 8th edition of American Joint Committee on Cancer staging system.
† No esophageal lesion was evident at the time of CRT as patients had recurrent esophageal cancer after surgical resection.

cfDNA concentrations, cfDNA fragmentation profiles, and genomic copy number variations

A total of 139 blood samples were obtained from 43 patients before treatment, 39 patients after induction chemotherapy, and 57 patients at the time of response evaluation after CRT. The median time differences between pretreatment and post-induction chemotherapy draws, the pretreatment and post-CRT draws, and the post-induction chemotherapy and post-CRT draws were 42.5, 117, and 75 days, respectively.

The median baseline cfDNA concentration was 0.0286 (range, 0.0128-0.1210) ng/μL in ESCC patients (n = 43), and 0.0224 (0.0102-0.0434) ng/μL in healthy controls (n = 97; $P = 0.0004$; Fig 1A). The median baseline FR-score was 10.31 (range 9.56-16.04) in ESCC patients and 9.71 (9.23-12.84) in healthy controls ($P < 0.0001$; Fig 1B).

WGS was successful in all cfDNA samples, with the median baseline I-score of 8.38 (range 7.50-10.76) in ESCC patients, and 8.02 (7.66-8.40) in healthy controls ($P < 0.001$; Fig 1C). A circos

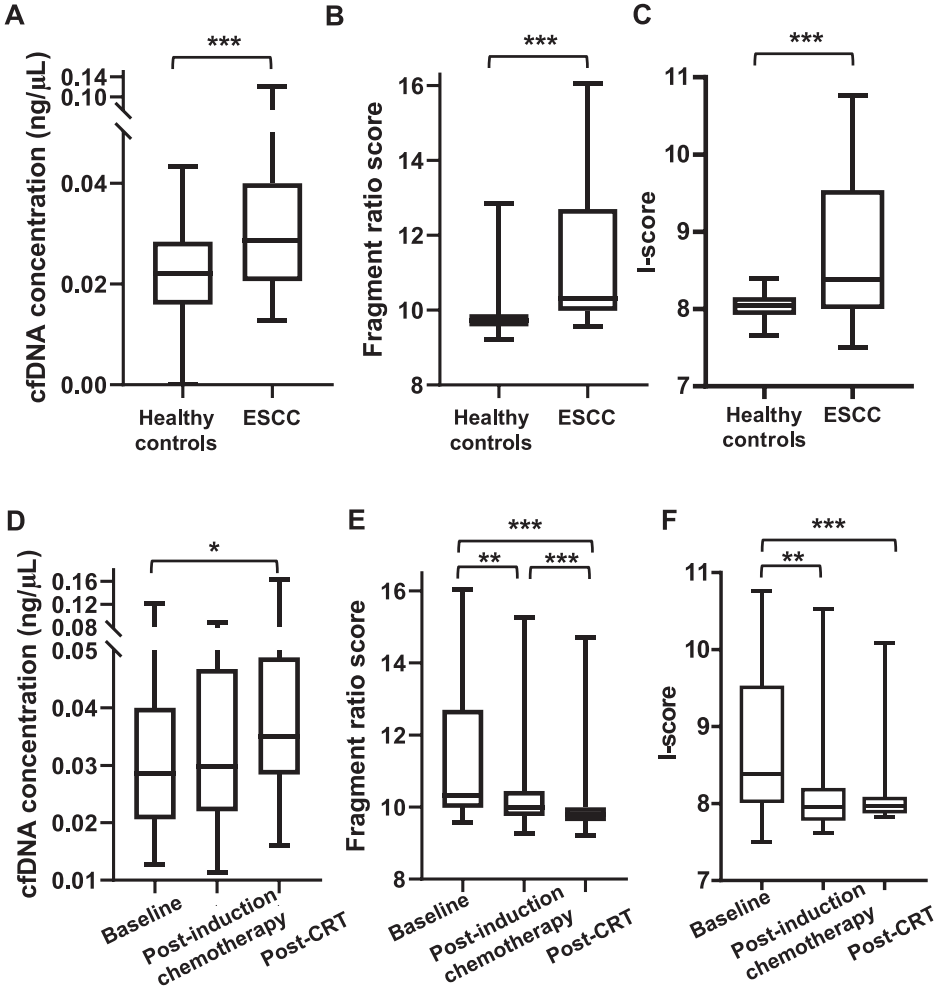

Fig. 1. (A) The concentration of cell-free DNA (cfDNA), (B) Fragment Ratio (FR)-score, and (C) I-score in healthy controls and esophageal squamous cell carcinoma (ESCC) patients. The baseline, post-induction chemotherapy, and post-chemoradiotherapy (CRT) (D) cfDNA concentrations, (E) FR-score, and (F) I-score in ESCC patients.
The box signifies the 75% (upper) and 25% (lower) quantiles and the horizontal line inside the box represents the median. The whiskers above and below the box represent the maximum and minimum values.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

plot from 108 ESCC patients shows the number of regions significantly deviated from euploidy (Supplementary Fig S1). GISTIC analysis[19] identified significantly recurrent focal amplifications at 3q26.32, 3q28, 7p11.2 (harboring EGFR), 8p11.22 (harboring FGFR1), 8q24.21, 9p24.1, and 11q13.3 (harboring Cyclin D1) regions. Oncogenes included in such recurrent regions were previously known as significantly altered regions in esophageal cancer.[20]

The median cfDNA concentration significantly increased after CRT (0.0350) compared to the baseline (0.0286, $P = 0.027$), although not after induction chemotherapy (0.0298; $P = 0.869$; Fig 1D). In contrast, median FR-score was significantly decreased after induction chemotherapy (9.984; $P = 0.003$) or CRT (9.783; $P < 0.001$) compared to the baseline (10.310; Fig 1E). Compared to post-induction chemotherapy, FR score further decreased after CRT ($P = 0.001$). Likewise, me-

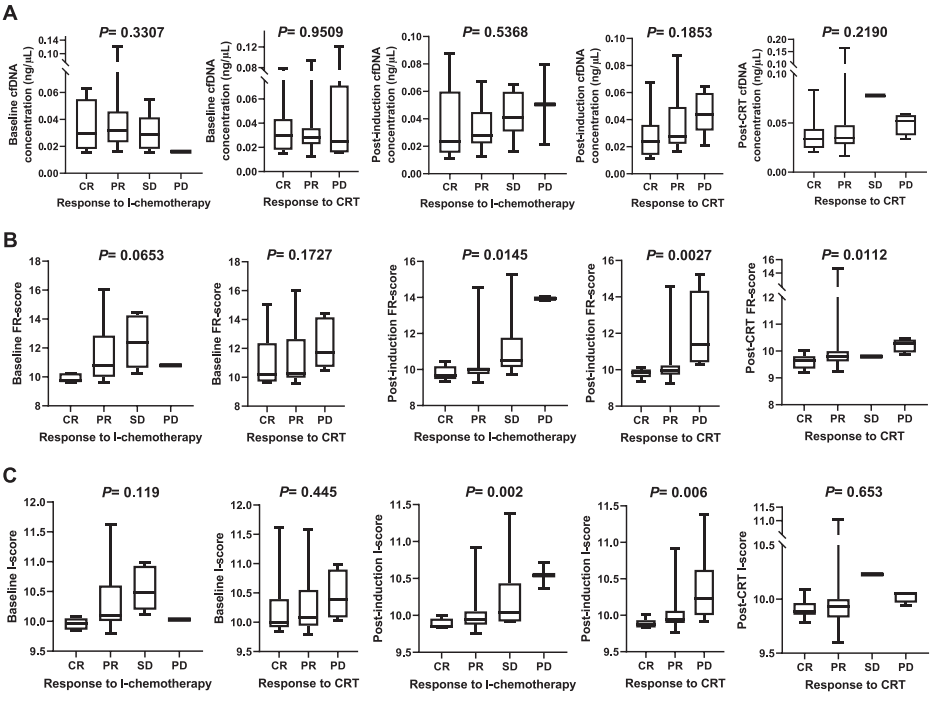

Fig. 2. Baseline, post-induction (I) chemotherapy, and post-chemoradiotherapy (CRT) (A) cfDNA concentrations, (B) FR-scores, and (C) I-scores according to responses to induction chemotherapy or chemoradiotherapy.
CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

dian I-score significantly decreased after induction chemotherapy (7.95; $P = 0.001$) or CRT (7.92; $P < 0.001$) compared to the baseline (8.38; Fig 1F).

FR-score significantly correlated with I-score (baseline, $r = 0.98$, $P < 0.001$; post-induction chemotherapy, $r = 0.94$, $P < 0.001$; and post-CRT, $r = 0.89$, $P < 0.001$), whereas cfDNA concentration did not correlate with either FR-score or I-score (Supplementary Fig S2). The correlation between FR-score and I-score appeared to be weaker after treatment, in particular, CRT ($r = 0.89$, $P < 0.001$).

Association between indices of cfDNA and treatment outcomes

Baseline and post-treatment cfDNA concentrations, FR-scores, and I-scores according to tumor responses are shown in Figure 2. The cfDNA concentrations before or after treatment were not associated with treatment outcomes (Fig 2A, Table S1). However, the baseline FR-score was significantly associated with metabolic response to induction chemotherapy ($P = 0.040$; Table 2). The FR-score-low group (scores below the median) was more likely to have metabolic CR than the FR-score-high group (above the median, 27.8% vs 0%; $P = 0.045$). Post-induction chemotherapy FR-score was also significantly associated with metabolic response to induction chemotherapy ($P = 0.0145$) and overall response to CRT ($P = 0.0027$). The FR-score-low group had a higher metabolic response rate after induction chemotherapy (94.7% vs 65.0%; $P = 0.044$), and overall response rate after CRT (100% vs 70.0%; $P = 0.020$) than the FR-score-high group. Post-CRT FR-score was also significantly associated with overall response to CRT ($P = 0.020$). The FR-score-low group was more likely to have clinical CR (34.5% vs 10.7%; $P = 0.033$) and CR/PR (100% vs 82.1%; $P = 0.023$) than the FR-score-high group after CRT.

Table 2
Association between FR-score and response to induction chemotherapy or chemoradiotherapy

Group of FR-score	Treatment response according to the evaluation time				*P*-value
	Metabolic response to induction chemotherapy				
Baseline	CR	PR	SD	PD	0.040
FR-score-low**	5 (27.8%)	12 (66.7%)	1 (5.6%)	0 (0%)	
FR-score-high**	0 (0%)	13 (72.2%)	4 (22.2%)	1 (5.6%)	
	Overall response to chemoradiotherapy				
Baseline	CR	PR	SD	PD	0.139
FR-score-low**	6 (26.1%)	16 (69.6%)	0 (0%)	1 (4.3%)	
FR-score-high**	3 (15.0%)	12 (60.0%)	0 (0%)	5 (25.0%)	
	Metabolic response to induction chemotherapy				
Post-induction chemotherapy	CR	PR	SD	PD	0.090
FR-score-low**	4 (21.1%)	14 (73.7%)	1 (5.3%)	0 (0%)	
FR-score-high**	1 (5.0%)	12 (60.0%)	5 (25.0%)	2 (10.0%)	
	Overall response to chemoradiotherapy				
Post-induction chemotherapy	CR	PR	SD	PD	0.017
FR-score-low**	6 (31.6%)	13 (68.4%)	0 (0%)	0 (0%)	
FR-score-high**	2 (10.0%)	12 (60.0%)	0 (0%)	6 (30.0%)	
	Overall response to chemoradiotherapy				
Post-chemoradiotherapy	CR	PR	SD	PD	0.020
FR-score-low**	10 (34.5%)	19 (65.5%)	0 (0%)	0 (0%)	
FR-score-high**	3 (10.7%)	20 (71.4%)	1 (3.6%)	4 (14.3%)	

** Patients were classified into the FR-score-low group and -high groups based on their FR-score value. The median value was the criteria for classifying FR-score-low and -high groups.
CR, Complete response; PR, partial response; SD, stable disease; PD, progressive disease

Table 3
Association between I-score and response to induction chemotherapy or chemoradiotherapy

Group of I-score	Treatment response according to the evaluation time				*P*-value
	Metabolic response to induction chemotherapy				
Baseline	CR	PR	SD	PD	0.003
I-score-low**	5 (27.8%)	12 (66.7%)	0 (0%)	1 (5.6%)	
I-score-high**	0 (0%)	13 (72.2%)	5 (27.8%)	0 (0%)	
	Overall response to chemoradiotherapy				
Baseline	CR	PR	SD	PD	0.160
I-score-low**	6 (27.3%)	15 (68.2%)	0 (0%)	1 (4.5%)	
I-score-high**	3 (14.3%)	13 (61.9%)	0 (0%)	5 (23.8%)	
	Metabolic response to induction chemotherapy				
Post-induction chemotherapy	CR	PR	SD	PD	0.113
I-score-low**	3 (15.0%)	16 (80.0%)	1 (5.0%)	0 (0%)	
I-score-high**	2 (10.5%)	10 (52.6%)	5 (26.3%)	2 (10.5%)	
	Overall response to chemoradiotherapy				
Post-induction chemotherapy	CR	PR	SD	PD	0.022
I-score-low**	5 (25.0%)	15 (75.0%)	0 (0%)	0 (0%)	
I-score-high**	3 (15.8%)	10 (52.6%)	0 (0%)	6 (31.6%)	
	Overall response to chemoradiotherapy				
Post-chemoradiotherapy	CR	PR	SD	PD	0.479
I-score-low**	8 (27.6%)	20 (69.0%)	0 (0%)	1 (3.4%)	
I-score-high**	5 (17.9%)	19 (67.9%)	1 (3.6%)	3 (10.7%)	

** Patients were classified into the I-score-low group and -high groups based on their I-score value. The median value was the criteria for classifying I-score-low and -high groups.
CR, Complete response; PR, partial response; SD, stable disease; PD, progressive disease

I-score was also associated with post-induction chemotherapy or post-CRT responses (Fig 2C, Table 3). The baseline I-score was significantly associated with metabolic response to induction chemotherapy ($P = 0.003$; Table 3). Post-induction chemotherapy I-score was significantly associated with metabolic response to induction chemotherapy ($P = 0.002$) and overall response to

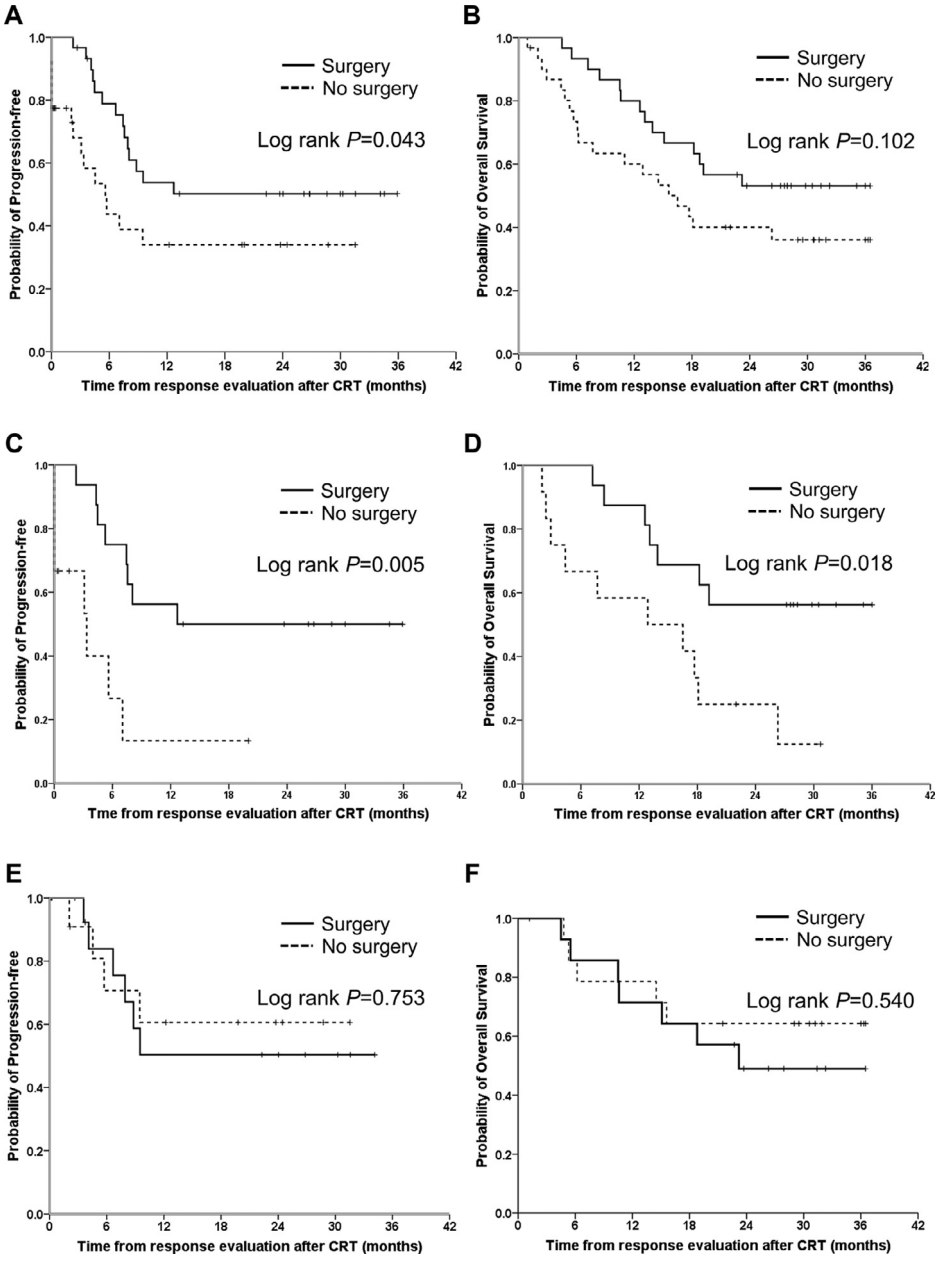

Fig. 3. Kaplan-Meier curves showing time to progression and overall survival according to treatment after chemoradiotherapy (surgery vs no surgery) (A, B) in all patients, (C, D) in patients with high post-chemoradiotherapy FR-score (above the median), and (E, F) in patients with low post-chemoradiotherapy FR-score (below the median).

CRT ($P = 0.006$; Fig. 3C). The I-score-low group had higher metabolic response rate after induction chemotherapy than the I-score-high group (95.0% vs 63.2%; $P = 0.020$).

In patients who received definitive CRT (n = 31), those with low baseline or post-treatments FR-score had longer TTP (median, with baseline FR-score, not reached vs 5.8 [95% confidence interval {CI} 0.6-11.0] months, $P = 0.026$; with post-induction FR-score, not reached vs 4.1 [0-10.2]

months, $P = 0.011$; with post-CRT FR-score, not reached vs 7.5 [3.3-11.7] months, $P = 0.008$) and OS (median, with baseline FR-score, not reached vs 17.0 [10.0-24.0] months, $P = 0.089$; with post-induction FR-score, not reached vs 13.9 [4.9-22.9] months, $P = 0.104$; with post-CRT FR-score, not reached vs 17.0 [1.7-32.3] months; $P = 0.02$), than those with high FR-score (Supplementary Fig S3). Also, in the same patient population, low baseline I-score was significantly associated with longer TTP (median, not reached vs 3.4 [95% CI, 0-8.7] months, $P = 0.019$) than those with high baseline I-score (Supplementary Fig S4). However, these differences of survival were not significant with post-treatments I-score (Supplementary Fig S4) or cfDNA concentration (data not shown).

After CRT, patients who received surgery had significantly better median TTP (not reached vs 5.6 [95% CI, 2.3-8.9] months; $P = 0.043$) and tended to have better median OS (not reached vs 16.5 [10.1-22.9] months; $P = 0.102$) than those who did not (Fig 3A and B). Notably, the impact of surgery on prognosis differed according to the post-CRT FR-score. While in the post-CRT FR-score-high group, patients who underwent surgery still had significantly better TTP (median, 12.7 vs 3.4 [95% CI, 0-7.5] months; $P = 0.011$) and OS (median, not reached vs 12.9 [0-27.8] months; $P = 0.018$) than those who did not (Fig 3C and D), the post-CRT FR-score-low group showed no differences in TTP ($P = 0.753$) and OS ($P = 0.540$) between patients with surgery and those without surgery (Fig 3E and F). These differential effects of surgery based on the cfDNA biomarkers after CRT were not observed in the cfDNA concentration or I-score (Supplementary Fig S5).

Discussion

For locally advanced esophageal cancer, aggressive trimodal therapy consisting of CRT and surgery is performed. However, surgical esophagectomy is associated with considerable morbidity and mortality, and decreased long-term quality of life.[21-23] Although the addition of neoadjuvant CRT to surgery demonstrated survival benefits compared to surgery alone in patients with locally advanced esophageal cancer, previous studies suggested that surgery could potentially be avoided in responding patients to CRT.[4,5,24] In phase III studies and our previous randomized study, addition of surgery after CRT resulted in improved locoregional control but was not translated to survival benefits.[4,5,25] However, the accurate clinical response to CRT in esophageal cancer is difficult to be evaluated using conventional imaging and endoscopy, and assessment methods and definition of response have varied according to studies. Novel biomarkers such as ctDNA are intriguing research area to screen good responders after CRT and to aid therapeutic decision making in post-CRT period.

In the present study, FR-score and I-score were calculated from plasma cfDNA reflecting fragment lengths and CNV of cfDNA, respectively. The baseline cfDNA concentration, FR-score and I-score were significantly higher in ESCC patients than in healthy controls, and the FR-score and I-score significantly decreased after induction chemotherapy or CRT, which may suggest these scores reflect tumor burden. However, ctDNA concentration increased after CRT, which may be confounded with inflammation caused by radiation therapy. Low baseline, post-induction chemotherapy and post-CRT FR-scores were significantly associated with improved treatment response. Post-induction chemotherapy I-score was also associated with treatment response. In patients who received definitive CRT, those with high FR-score showed shorter TTP and OS than those with low FR-score, and the difference was statistically most significant with the post-CRT FR-score. Most importantly, patients with surgery after CRT showed significantly longer survival than patients without surgery in the FR-score-high group, while there was no survival benefit with surgery in the FR-score-low group. ctDNA is increasingly studied as a biomarker in various cancers due to its potential to identify genomic alterations in tumor tissues, correlate with tumor burden, detect minimal residual disease and predict treatment outcomes and prognosis.[15,26-32] Particularly, recent studies have shown that ctDNA enables prediction of relapse and chemotherapeutic efficacy, and detection of minimal residual disease after CRT in esophageal cancer patients.[29,30] However, powerful methods and techniques including deep sequencing are

required to distinguish ctDNA from other cfDNA by identifying tumor-specific genetic aberrations.[31] Fragment size analysis and selective sequencing of specific fragment sizes can boost ctDNA detection and provide an alternative to deeper sequencing of cfDNA for clinical applications.[11,16] In a previous study, "DNA evaluation of fragments for early interception (DELFI)" score was obtained utilizing machine-learning process of cfDNA fragmentation profiles of cancer patients and healthy individuals and could be used in early detection and monitoring of cancer. In this study, we developed FR-score which is easily calculated and reflects the fragmentation pattern of cfDNA. Higher FR-score indicates higher proportion of short fragments of cfDNA, which consequently means higher proportion of ctDNA fragments in cfDNA.[8-11]

Patients in the FR-score-high group showed poorer responses to CRT. Also, patients with high FR-score after CRT showed poor post-CRT survival and gained benefit from surgery after CRT, which suggests that high post-CRT FR-score predicts the possibility of residual tumor after CRT. Since patients with high post-CRT FR-score were more likely to have poor treatment outcomes, adding treatment such as surgery after CRT would be helpful for these patients. Currently, there is no guideline related to additional treatment strategy after definitive CRT. Thus, FR-score is potentially a novel guiding biomarker in combination with post-CRT clinical response to predict residual tumor burden, treatment response, and survival after CRT, and to decide post-CRT treatment strategy including whether to add morbid surgery. FR-score has advantage in that it uses a relatively simple and inexpensive method. However, limitations in this approach exist. Classifying patients into the FR-score-low and -high groups based on the median FR-score may have limited sensitivity in predicting residual tumor burden after CRT. More sensitive methods such as tumor-guided personalized deep sequencing would be required to increase accuracy, despite the high cost.

In the present study, patients with high CNV rates, as represented by high I-score, showed poorer treatment response, but were not associated with survival outcome after CRT. Although a previous study of advanced hepatocellular carcinoma patients receiving sorafenib showed that I-score could be a biomarker predicting survival outcomes,[18] I-score did not appear adequately sensitive to predict survival outcomes in this study. This result may be due to different type of cancer and treatment, and different disease status between 2 studies. Esophageal cancer has shown a lower CNV rate than hepatocellular carcinoma does, and this study included locally advanced esophageal cancer, which was a less advanced disease status than included in the previous study.[20,33]

In the present study, cfDNA concentration was not significantly associated with treatment outcomes. This result could be attributed to low sensitivity and false positivity due to inflammation.[18] cfDNA concentration is known to be elevated in conditions of increased cell death, including inflammation.[34,35] Since inflammation and cancer necrosis occur after CRT, cfDNA concentration may not be a good biomarker of treatment outcome after CRT.

In conclusion, FR-score from cfDNA analysis in patients who receive CRT for esophageal cancer could be a novel biomarker to predict response, residual tumor burden and survival after CRT, and consequently, decide post-CRT treatment strategy including whether to add morbid surgery. Since the present study elucidated a potential biomarker for locally advanced ESCC, further well-designed prospective studies with appropriate statistical power for predefined endpoints are required for validation.

Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.currproblcancer.2020.100685.

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