

Assessment of a direct fluorescence-based cell-free DNA assay for evaluating the extent of metastatic dissemination: An exploratory study

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Abstract. Fluorescence-based cell-free DNA (cfDNA) assays quantify nucleic acids without the need for DNA extraction and amplification. The present study investigated whether cfDNA levels differed between patients with active cancer and those with no evidence of disease (NED). Blood samples from patients with metastatic cancer (Patient group) and from individuals with NED (patients that had completed curative-intent treatment and remained recurrence free for ≥ 1 year; NED group) were used to quantify cfDNA levels using PicoGreen™ reagent. In total, 124 participants were prospectively enrolled between January 2020 and August 2021 (Patient group, n=72; NED group, n=52). The median age was 69 years (range: 27-83 years) in the Patient group and 65 years (range: 34-81 years) in the NED group. In the Patient group, the pancreatobiliary (n=32, 44.4%), genitourinary (n=20, 27.7%) and gastrointestinal (n=6, 8.3%) sites were the most common primary cancer sites. The median cfDNA value in the Patient group (9.80 $\mu\text{g/ml}$, IQR 8.63-11.08) was significantly higher than that in the NED group (8.40 $\mu\text{g/ml}$, IQR 7.75-9.10, $P<0.001$). In addition, the median cfDNA levels were significantly higher in patients with a high extent of metastatic

dissemination (defined as metastases in three or more organs) than in those with a low tumor extent (10.70 $\mu\text{g/ml}$, IQR 9.35-12.4 vs. 9.45 $\mu\text{g/ml}$, IQR 8.48-10.85, respectively; $P=0.027$). Notably, there was no significant correlation between cfDNA and conventional tumor marker levels, including carcinoembryonic antigen and carbohydrate antigen 19-9 levels ($P=0.468$ and $P=0.134$, respectively). These findings indicated that cfDNA levels were elevated in patients with metastatic cancer compared with those with NED, particularly in those with a higher extent of metastatic dissemination. Additionally, cfDNA levels were not correlated with those of conventional tumor markers. These cross-sectional exploratory data highlight the potential utility of fluorescence-based cfDNA assays in assessing the extent of metastatic dissemination, and provide a foundational rationale for future longitudinal studies investigating treatment monitoring and prognosis.

Introduction

Cell-free DNA (cfDNA), a biomarker in tumor-related clinical settings, reflects physiological and pathological processes (1). cfDNA comprises fragmented DNA in body fluids arising from apoptosis or necrosis (2) and is elevated in cancer (3,4). cfDNA quantification is increasingly recognized as useful for cancer diagnosis and prognosis as well as for monitoring therapeutic response and postoperative residual disease (1-3,5,6).

Conventional cfDNA quantification (via polymerase chain reaction) requires DNA extraction and amplification, which are tedious, time-consuming, and costly (7). Protocol and reagent variations hinder standardization, making interlaboratory and interoperator comparisons difficult. Conversely, fluorescence-based cfDNA assays are simpler, faster, and cost-effective (8). Such assays correlate with polymerase chain reaction-based quantification (9) and have revealed significant changes in cfDNA levels that reflect anticancer treatment efficacy (5,10).

cfDNA typically contains a small fraction of circulating tumor DNA (ctDNA), which creates high background noise and limits the detection of tumor-derived signals (1,11,12).

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Abbreviations: cfDNA, cell-free DNA; NED, no evidence of disease; BSA, body surface area

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Active cancer involves increased cell turnover, apoptosis, and necrosis within the tumor and its microenvironment (13). Although cfDNA is primarily released from non-tumor tissues, elevated cfDNA levels may still reflect cancerous processes, and the clinical utility of cfDNA assays lies in the continuous monitoring of dynamic changes rather than absolute cut-off values (1). cfDNA assays are particularly useful for evaluating the extent of metastatic dissemination and monitoring of treatment responses in advanced settings. There is a lack of well-designed, prospective studies evaluating fluorescence-based cfDNA assays, and the majority of the evidence is derived from retrospective analyses (3,5).

We assessed the fluorescence-based cfDNA assay performance (accuracy and reproducibility) and cfDNA stability based on storage temperature, sample processing time, and freeze-thaw effects to confirm that the cfDNA values remained stable under these conditions (10). We demonstrated that applying these assays to frozen samples from biobanks is feasible within the practical constraints of real-world hospital settings. In the future, we will conduct a comparative study between patients with cancer and healthy controls to evaluate the diagnostic accuracy of fluorescence-based cfDNA assays for cancer detection.

Materials and methods

Study population. Blood samples were prospectively collected between January 2020 and August 2021 at Pusan National University Yangsan Hospital (Yangsan, Republic of Korea) from patients with any type of cancer and confirmed distant metastasis (Patient group) and from individuals who had completed curative treatment for localized cancer and had been free of residual disease for at least 1 year [no evidence of disease (NED) group]. The Patient group included patients who had evident metastatic disease at the time of enrollment. Patients who had no definite residual disease after metastasectomy or those with an uncertain recurrence status were excluded. Patients were also excluded if they had a clinically evident active infection, had received any anti-cancer treatment, including chemotherapy, radiotherapy, surgery, or palliative surgery, within the preceding 6 months, or were receiving immunosuppressive agents, including corticosteroids, or herbal medications. The NED group consisted of individuals who had completed curative treatment for localized cancer and had no evidence of residual or recurrent disease during follow-up. Only individuals with no evidence of recurrence at the time of blood collection and with C-reactive protein, lactate dehydrogenase, white blood cell count, and platelet count within the normal ranges were included. This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Pusan National University Yangsan Hospital (approval no. 042019024). Written informed consent was obtained from all participants before enrollment in the study.

Blood sample collection. Peripheral blood samples were collected from all participants into ethylenediaminetetraacetic acid tubes (to prevent coagulation) using standard venipuncture techniques and processed (3,000 × g for 10 min at room temperature) within 1 h to obtain plasma, which minimizes *in vitro* leukocyte lysis. Plasma samples were

visually inspected, and those with macroscopic hemolysis were excluded to prevent contamination by genomic DNA. Plasma samples were aliquoted into microcentrifuge tubes and stored at -80°C in a biobank until further analysis.

Quantification of fluorescence-based cfDNA. To prevent experimental or observational bias, all biobanked plasma samples were labeled with anonymized numeric barcodes, ensuring that laboratory personnel were completely blinded to the clinical groups (Patient vs. NED). Furthermore, samples from both groups were randomly distributed across the assay plates. Fluorescence-based cfDNA was quantified directly using Quant-iT PicoGreen™ dsDNA Reagent (P7584, Invitrogen, USA) without the need for prior DNA extraction or amplification steps. Plasma samples were diluted at a 1:50 ratio with Tris-ethylenediaminetetraacetic acid buffer (V6231; Promega, USA) before quantification. All standards and samples were dispensed into wells of a 96-well microplate (100 µl of sample aliquot per well). The samples were stained with PicoGreen reagent by adding 100 µl of the reagent to each sample in black 96-well plates (SPL30296, SPL, Korea), followed by a 400-fold dilution of the final mixture. Every standard and plasma sample was measured in duplicate (two independent assays per sample). The fluorescence intensity was quantified using a black 96-well microplate reader (Spark, Tecan, Switzerland) at excitation and emission wavelengths of 485 and 535 nm, respectively, and the average of the two replicate measurements was used as the final summarized value for all statistical analyses. Information regarding the basic performance characteristics and validation of this fluorescence-based cfDNA assay has been reported previously (2,14). Fluorescence-based cfDNA assays were performed on all biobanked plasma samples on the same day.

Statistical analysis. Continuous variables are expressed as means ± standard deviations or medians with interquartile ranges (IQR), depending on the distribution. The Mann-Whitney U test was used to compare cfDNA levels between the Patient and NED groups. Spearman's rank correlation coefficient was used for all correlation analyses. Because the distributions of cfDNA and tumor markers exhibited severe positive skewness with extreme outlier values, this non-parametric method was purposefully selected to robustly evaluate monotonic relationships without being unduly skewed by extreme high-end values. To adjust for potential confounders including age, sex, and body surface area (BSA), a multivariable analysis (analysis of covariance) was performed. Prior to this analysis, cfDNA values were natural log-transformed to satisfy the assumption of normality. $P < 0.05$ was considered to indicate a statistically significant difference. All calculations were performed using SPSS for Windows (version 21.0; IBM Corp., Armonk, NY, USA).

Results

Patient characteristics. Between January 2020 and August 2021, a total of 124 participants were enrolled at Pusan National University Yangsan Hospital, (Patient group $n=72$; NED group $n=52$), with a median age of 69 years (range 27-83) in the Patient group, while that in the NED group was 65 years (range 34-81). Sex distribution and BSA were similar

Table I. Baseline characteristics of the Patient and NED groups.

Characteristic	Patient group (n=72)	NED group (n=52)
Median age, years (range)	69 (27-83)	65 (34-81)
Sex, n (%)		
Male	46 (63.9)	32 (61.5)
Female	26 (36.1)	20 (38.5)
Type of cancer, n (%)		
Gastrointestinal cancer	6 (8.3)	19 (36.5)
Pancreatobiliary cancer	32 (44.4)	23 (44.2)
Genitourinary cancer	20 (27.7)	4 (7.7)
Lung cancer	4 (5.6)	0 (0.0)
Gynecologic cancer	4 (5.6)	1 (1.9)
Others ^a	6 (8.3)	5 (9.6)
Median duration of NED, months (95% CI)	N/A	81.0 months (64.8-97.3)
Mean $\pm$ SD BSA, m ²	1.61 $\pm$ 0.17	1.69 $\pm$ 0.18
Number of metastatic sites, n (%)		N/A
1	35 (48.6)	
2	23 (31.9)	
≥ 3	14 (19.4)	
Mean time from blood collection to cfDNA analysis, months (range)	12.7 (2.6-23.5)	11.4 (2.2-21.6)

^aOthers include sarcomas, neuroendocrine carcinomas, and germ cell tumors. BSA, body surface area; cfDNA, cell-free DNA; NED, no evidence of disease; SD, standard deviation.

between the two groups. The distribution of cancer types was pancreatobiliary (44.4%), genitourinary (27.7%), gastrointestinal (8.3%), lung (5.6%), and gynecological (5.6%). The NED group cancer type distribution was pancreatobiliary (44.2%), gastrointestinal (36.5%), genitourinary (7.7%), and gynecological (1.9%). At baseline, 35 patients (48.6%) presented with a single metastatic site, whereas 23 (31.9%) and 14 (19.4%) had two and three or more metastatic sites, respectively. The disease-free duration in the NED group was 81.0 months (95% confidence interval, 64.8-97.3). The mean storage duration was 12.7 months (95% CI, 11.1-14.2; range, 2.6-23.5) in the Patient group and 11.4 months (95% CI, 10.1-12.7; range, 2.2-21.6) in the NED group, with no significant difference between the groups ($P=0.208$) (Table I). Furthermore, there was no significant correlation between storage duration and measured cfDNA levels across the entire cohort (Spearman's $\rho=0.146$, $P=0.098$).

Differences in cfDNA levels between the Patient and NED groups. cfDNA levels were significantly higher in the Patient group than in the NED group. As shown in Fig. 1, the median cfDNA level was 9.80 $\mu\text{g/ml}$ (IQR 8.63-11.08) in the Patient group and 8.40 $\mu\text{g/ml}$ (IQR 7.75-9.10) in the NED group (95% CI 0.97-2.11, $P<0.001$). Importantly, the difference in cfDNA levels between the Patient and NED groups remained highly statistically significant even after adjusting for age, sex, and BSA in the multivariable analysis (adjusted $P<0.001$; Table SI).

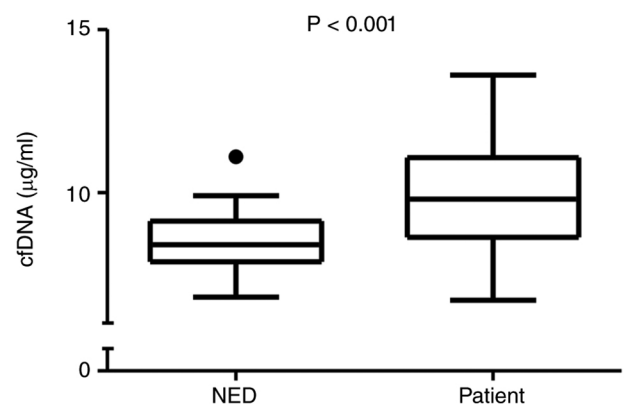


Figure 1. Difference in cfDNA levels between the Patient group and the NED group. Median cfDNA levels were significantly higher in the Patient group than in the NED group ($P<0.001$). cfDNA, cell-free DNA; NED, no evidence of disease.

cfDNA levels in relation to extent of metastatic dissemination, tumor markers, and baseline characteristics. A significant association was observed between cfDNA levels and extent of metastatic dissemination (Fig. 2). Patients with three or more metastatic organs had significantly higher cfDNA levels than those with one or two metastatic sites [median, 10.70 $\mu\text{g/ml}$ (IQR, 9.35-12.40) vs. 9.50 $\mu\text{g/ml}$ (IQR, 8.50-10.80), 95% CI 0.24-3.55, $P=0.027$]. Conversely, there was no significant correlation between cfDNA and tumor marker

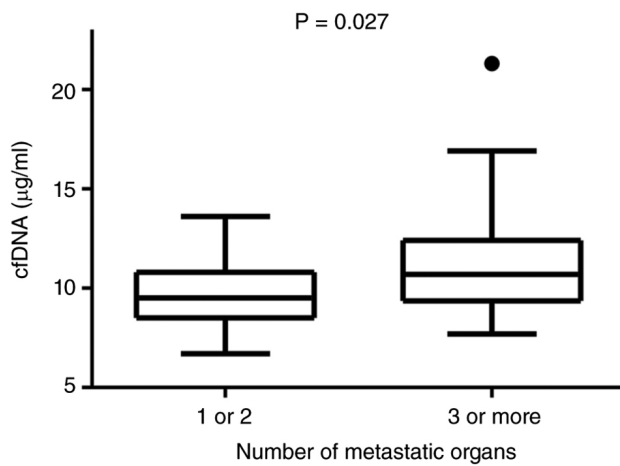


Figure 2. cfDNA levels according to extent of metastatic dissemination. Median cfDNA levels were higher in patients with three or more metastatic organs than in those with one or two metastatic organs ($P=0.027$). cfDNA, cell-free DNA.

levels [carcinoembryonic antigen (CEA), $n=104$; carbohydrate antigen (CA)19-9, $n=95$] in the Patient group (Fig. 3). Although cfDNA levels significantly increased with higher extent of metastatic dissemination, these levels were not associated with conventional tumor markers.

The relationship between cfDNA levels and baseline characteristics was also assessed (Fig. 4). cfDNA levels showed a weak but statistically significant increase with advancing age ($P=0.038$). Additionally, no significant differences in cfDNA levels were observed between male and female patients [median, $9.20 \mu\text{g/ml}$ (IQR, $8.40\text{--}10.10$) vs. $8.60 \mu\text{g/ml}$ (IQR, $7.80\text{--}9.80$), 95% CI $-0.66\text{--}0.97$, $P=0.705$]. Similarly, no significant association was observed between cfDNA levels and BSA.

Discussion

Fluorescence-based cfDNA levels were significantly higher in patients with metastatic cancer than in those with NED, with a notable association between cfDNA levels and extent of metastatic dissemination. These findings revealed that the fluorescence-based cfDNA assay is a biomarker in cancer management, particularly for assessing the extent of metastatic dissemination, tracking therapeutic response, detecting minimal residual disease postoperatively, and conducting surveillance. This assay is a quantitative tool that cannot be applied to individual genomic profiles, making it unsuitable for precision medicine approaches that rely on tumor mutation profiling, such as ctDNA-based tumor genotyping (15,16). Nevertheless, we demonstrated a positive correlation between cfDNA levels and a high extent of metastatic dissemination. Associations between cfDNA levels and clinical variables (BSA and sex) were observed. These findings suggest that although a fluorescence-based cfDNA assay may not be directly applicable to precision treatment strategies or to establish a universal absolute cut-off value for clinical use, it may still have clinical utility when used for serial measurements in individual patients. For example, conducting serial cfDNA measurements and correlating their impact on tumor dynamics and response to treatment may facilitate treatment optimization

and guide timely adjustment of treatment, such as the escalation or de-escalation of therapies. Similarly, ctDNA assays, which traditionally focused on identifying actionable mutations, are increasingly being used in longitudinal monitoring to evaluate clinical implications based on dynamic changes in ctDNA levels (17-19). In this context, fluorescence-based cfDNA assays offer advantages for repeated measurements because of their low interlaboratory variability and cost-effectiveness. In the era of immunotherapy, where cure rates improve through the eradication of micrometastatic disease before the onset of clinically evident disease (20), fluorescence-based cfDNA assays may be valuable for tracking disease progression and screening for minimal residual disease in postoperative settings or during long-term surveillance.

It is important to consider the biological mechanism underlying the direct PicoGreen quantification. Recent studies indicate that a substantial fraction of cfDNA may circulate within exosomes or extracellular vesicles (21), this remains a topic of debate; other studies conflict with this finding, suggesting that vesicle-encapsulated DNA is much less abundant than free, non-vesicular cfDNA (22). Furthermore, because our simplified protocol does not employ a stringent lysis step, it is unclear whether the PicoGreen dye is able to detect and measure vesicular cfDNA. Because we did not experimentally assess the vesicular versus non-vesicular fractions of the detected cfDNA, the ability of PicoGreen to access extracellular vesicles, or the specific cellular mechanisms responsible for cfDNA release, further investigations are required to definitively determine the biological source of the strong fluorescence signals observed in patients with advanced metastasis.

We did not observe significant correlations between cfDNA levels and conventional tumor markers commonly used in clinical practice. However, these results should be interpreted cautiously. Because our cohort comprises diverse cancer types where CEA and CA19-9 are typically elevated only in specific subgroups, such as gastrointestinal or pancreaticobiliary malignancies, a global correlation analysis may obscure tissue-specific relationships. Therefore, the lack of broad correlation highlights the distinct biological properties of total cfDNA compared to specific protein markers, rather than invalidating either modality. This finding underscores the unique potential of cfDNA as a complementary biomarker, which may offer distinct advantages over tumor markers. The short half-life of cfDNA in the circulation (approximately 1 h) can be advantageous for assessing the real-time extent of metastatic dissemination in response to therapy, in contrast to employing numerous tumor markers, which have half-lives of days to weeks (18). As cfDNA monitoring becomes more widely available, its potential to detect early signs of treatment response or disease progression (particularly in surveillance settings) may become increasingly important for clinical decision-making.

Despite the promising findings of this study, it had several limitations. First, all patients included in the Patient group were diagnosed with metastatic cancer, and none of the patients with early-stage or localized disease were enrolled. A comparison between patients with localized cancer and those with NED would have been more appropriate to evaluate the role of cfDNA in detection of recurrence or screening for minimal residual disease. Furthermore, because this exploratory study

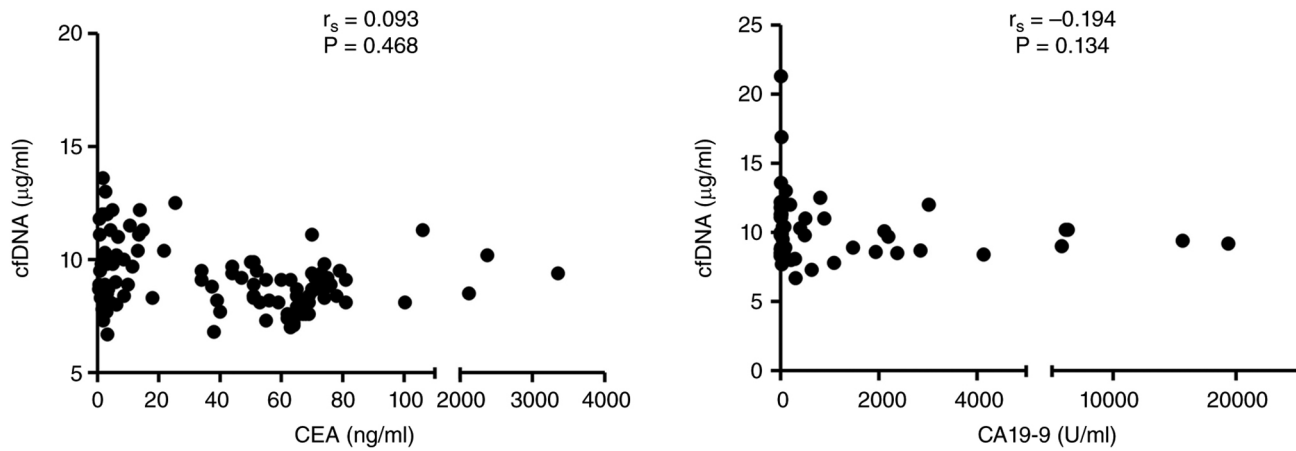


Figure 3. Correlation between cfDNA and tumor marker levels in the Patient group. The scatter plots showed no significant correlation between cfDNA levels and conventional tumor markers, as indicated by the low correlation coefficients and non-significant P-values. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; cfDNA, cell-free DNA.

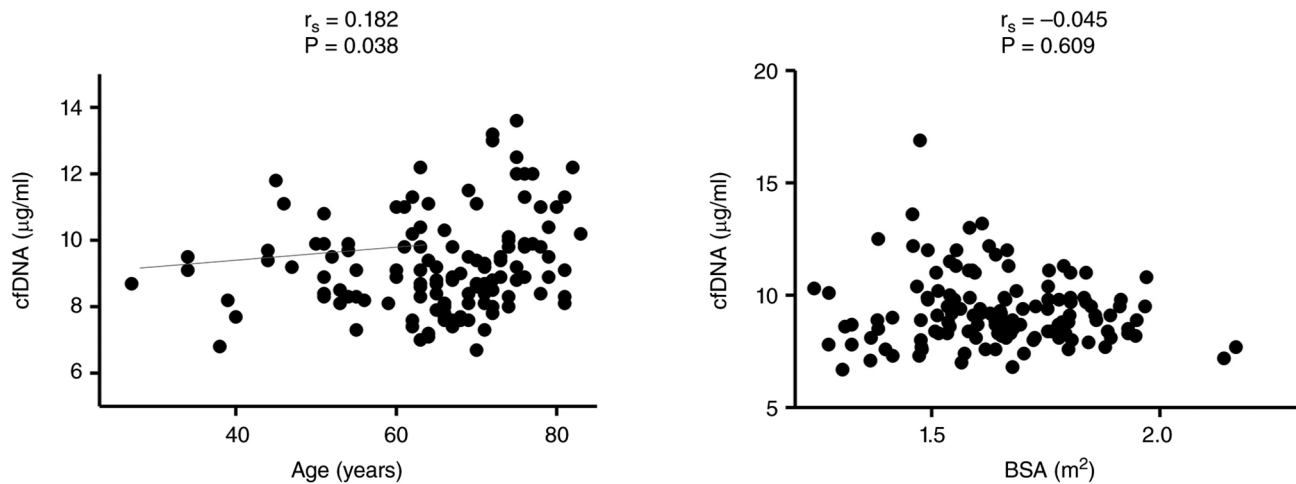


Figure 4. Association between cfDNA levels and baseline characteristics in the overall study population. The analysis revealed that cfDNA levels were significantly associated with age, while no significant correlation was observed with BSA. BSA, body surface area; cfDNA, cell-free DNA.

compared a highly selected cohort (metastatic vs. NED), we omitted receiver operating characteristic (ROC) analyses and diagnostic cut-off derivations to avoid artificially inflating diagnostic performance. Other limitations include the inability to perform multivariable adjustments for cancer heterogeneity between the two groups. Specifically, because the NED group intrinsically lacks a concurrent active cancer type, including cancer type as a covariate creates a perfect collinearity issue, rendering such a mathematical adjustment impossible. Furthermore, there remains the potential influence of unmeasured clinical confounders (e.g., inflammation, infections, or renal dysfunction) on total cfDNA levels. Additionally, our reliance on single-time-point assessments precludes the evaluation of dynamic cfDNA changes over time. Consequently, this exploratory study was intended to assess the potential clinical utility of cfDNA rather than to derive a cut-off value for immediate clinical application, and therefore does not establish clinical thresholds. However, the prospective design and clear demonstration of differences in cfDNA levels between cancer patients and NED individuals—as well as differences according to extent of metastatic dissemination—provide

evidence supporting the potential utility of fluorescence-based cfDNA assays. Future studies with longitudinal designs and serial measurements are warranted to further elucidate the clinical utility of this approach. The logical next step following this cross-sectional proof-of-concept study is to evaluate its utility in longitudinal monitoring. A prospective study is currently underway to investigate whether serial measurements of direct cfDNA levels in patients with advanced-stage cancer can rapidly reflect tumor response to systemic therapies [NCT05227950]. In addition, future prospective studies evaluating serial cfDNA measurements after curative treatment will be needed to determine their potential utility for the early detection of disease recurrence.

In conclusion, herein, we provide evidence supporting the potential of fluorescence-based cfDNA assays to noninvasively assess cfDNA levels and to support the assessment of the extent of metastatic dissemination. While our current findings are limited to cross-sectional exploratory evidence, they establish a rationale for future longitudinal investigations to validate the assay's utility in monitoring treatment responses and predicting clinical outcomes. Future studies exploring the

potential of these assays are warranted to validate our findings and further define their clinical potential in oncology.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JJK, YL and KP contributed to the conceptualization of the study. JJK, YL and YJH were responsible for data collection, experimentation and investigation. Formal analysis and the writing of the original draft were performed by JJK and YL. KP acquired the funding for the study. All authors (JJK, YL, YJH and KP) contributed to the review and editing of the manuscript. JJK and YL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Pusan National University Yangsan Hospital (ethical approval no. 04-2019-024; Yangsan, Republic of Korea). Written informed consent was obtained from all participants prior to their enrollment and any sample collection procedures.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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