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RESEARCH ARTICLE

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Analysis of cell-free circulating DNA fragment size and level in patients with lumbar canal stenosis

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Abstract

Cell-free circulating DNA (cfDNA), extracted by liquid biopsy, has been studied as a noninvasive biomarker for various diseases. The potential of cfDNA fragment size and level as a marker in lumbar canal stenosis (LCS) patients has never been studied. We investigated whether cfDNA is a biomarker of low back pain, leg pain, leg numbness severity in patients with an LCS. Blood samples were obtained from patients with LCS (n = 22) before and immediately after spinal surgery. Plasma DNA was isolated and examined for cfDNA fragment size and concentration. A cohort of healthy volunteers (n = 5) constituted the control group. The cfDNA fragment size tended to be shorter in patients than in healthy controls, but this difference was not significant ($P = .186$). cfDNA level was significantly higher in LCS patients (mean 0.614 ± 0.198 ng/μL, range 0.302-1.150 ng/μL) than in healthy controls (mean 0.429 ± 0.064 ng/μL, range 0.366-0.506 ng/μL) ($P = .008$). cfDNA level correlated positively with average pain ($r = .435$, $P = .026$) and leg numbness ($r = .451$, $P = .018$). cfDNA fragment size did not differ from before to after surgery, but cfDNA level increased postoperatively in patients with LCS. This was the first study investigating whether cfDNA fragment size and level are associated with pain in patients with LCS. Our findings suggest that cfDNA level may be an objective indicator of pain and surgical invasiveness in patients with LCS.

KEYWORDS
cell-free circulating DNA, lateral lumbar interbody fusion, low back pain, lumbar canal stenosis, numeric rating scale

1 | INTRODUCTION

Low back pain (LBP) is among the 10 most frequent reasons for visiting a primary care practitioner across all adult age groups.[1] In

Abbreviations: cfDNA, cell-free circulating DNA; CRP, C-reactive protein; CT, computed tomography; LBP, low back pain; LDD, lumbar degenerative disc disease; LN, leg numbness; LP, leg pain; MRI, magnetic resonance imaging.

industrialized countries, the lifetime prevalence of LBP is 70% to 90%, and the 1-year prevalence is 15% to 45%.[2] Clinically, LBP is caused by multiple triggers, but the most common etiologies of LBP include lumbar canal stenosis (LCS). LCS is a common disease in the elderly. The number of LCS patients who complain of LBP, leg pain (LP), and/or leg numbness (LN) increases yearly due to an increase in the elderly. LCS management comprises various intervention strategies, including surgery and nonmedical interventions such as exercise, behavioral therapy, and alternative therapies.

Pharmacological intervention is the most frequently recommended intervention for LBP.[3] In addition to nonsteroidal anti-inflammatory drugs and muscle relaxants, opioids, tramadol, benzodiazepines, and gabapentin (for radiculopathy) have now been added as possible pharmacological interventions. Spine surgeons are prescribing drugs that are likely useful for symptomatic treatment while considering the disease's pathophysiology.

The severity of pain is evaluated according to the therapeutic effects of treatment, whose effectiveness is based on subjective evaluation and, at present, there are no biomarkers for evaluating pain objectively. Various evaluation scales, such as the visual analog scale and numeric rating scale (NRS), evaluate pain. Basic research using animal models is limited by the inability to identify animal pain. Objective evaluation with a measuring device or biomarker that can measure pain is required; however, the lack of a biomarker to quantify pain is a barrier to pain research. Cell-free circulating DNA (cfDNA) is generally found in small amounts in the blood of healthy people. Elevated cfDNA levels have been reported in patients with malignant or autoimmune diseases, myocardial infarction, and trauma[4-6] and suggested that the release of cfDNA into the circulation may be useful, although nonspecific, a marker of tissue injury. The use of cfDNA fragment size and level as a potential marker in LCS patients has not been studied. Therefore, the purpose of this study was to investigate the potential of cfDNA levels as a biomarker for pain severity in patients with LCS.

2 | MATERIALS AND METHODS

The Committee approved the study on Ethics and the Institutional Review Board of Tokai University School of Medicine, the House Clinical Study Committee, and the Profit Reciprocity Committee (20R-263). The study was conducted following the principles outlined in the Helsinki declaration.[7]

All the participants provided informed written consent for the provision of blood sampling and clinical data.

2.1 | Participants

The inclusion criteria included patients 20 years of age or older and the need for surgery in the lumbar spine (L1-L2 to L5-S1) to treat LCS. All patients were diagnosed based on a detailed history, neurological and radiographic examinations, myelogram results, computed tomography (CT) scans after myelography, and/or magnetic resonance imaging (MRI).

In brief, participants were eligible if they had undergone spinal surgery, been diagnosed with a degenerative spinal disease based on physical and imaging findings, had received sufficient explanation about participating in this study, and had voluntarily provided written consent.

The exclusion criteria included severe mental illness, difficulty standing or moving because of severe impairment of paralysis, prior (within 5 years) or current cancer diagnosis, suspect a current infection, or the presence of a condition considered by the principal investigator to be a contraindication for inclusion in this study.

The authors provided information to the patients about the use of samples obtained during surgery, and patients were asked to read and sign the consent forms before surgery. Demographic data and clinical data were obtained after inclusion in the study.

Between October 2020 and April 2021, blood was collected from 22 patients (14 males, 8 females; age, 71.3 ± 8.1 years) diagnosed with LCS accompanied by LBP, LP, and/or LN and hospitalized for surgical purposes. Blood samples were collected from five healthy volunteers (four males, one female; age, 38.0 ± 7.8 years) who had never experienced LBP and were selected as the control group. Table 1 summarizes surgical records for LCS patients and the C-reactive protein (CRP) data on the first day after surgery.

2.2 | Procedures

A 10 mL blood sample was collected in Streck Cell-Free DNA BCT (Streck, La Vista, Nebraska). In the LCS patients, blood samples were collected immediately after induction of general anesthesia (baseline) and immediately after surgery.

Whole blood was separated from Streck Cell-Free DNA BCT by centrifugation at 1700*g* for 10 minutes at 25°C. The upper plasma layer was removed, transferred to a new conical tube, and centrifuged at 5000*g* for 10 minutes. Plasma samples were stored at −80°C until use. C-reactive protein (CRP) concentration was measured in postoperative blood samples as an indicator of the invasiveness of the surgery.

2.3 | Determination of cfDNA fragment size and level

The method uses the only 1 μL of DNA from plasma for one test, is very sensitive, with a dynamic range of 0 to 400 ng/μL human genomic DNA, and yields highly reproducible results. Qubit 3.0, with a dsDNA assay kit, was used to measure the total amount of genomic DNA.

cfDNA fragment size was determined for each sample with an Agilent 2100 Bioanalyzer and the Agilent High Sensitivity DNA chip, according to the manufacturer's instructions. The fragment size of cfDNA was determined with the Agilent 2100 Bioanalyzer software and defined as the main peak mode (corresponding to one nucleosome plus linker, derived from apoptotic cells) electropherogram. cfDNA level was performed using the Qubit fluorometer 3.0 (Invitrogen, Life Technologies) combined with the Qubit dsDNA HS

TABLE 1 Patients characteristics

No	Sex	Age (year)	Levels treated	No. of Levels	Diagnosis	Average OP time (min)	Average blood loss (ml)	Average length of stay (days)	First-post-OP-CRP (g/dL)
1	F	82	L4/5	1	LCS	78	99	20	5.04
2	M	57	L3/4/5	2	LCS	114	48	21	1.51
3	F	63	L3/4/5	2	LCS	293	695	20	3.54
4	M	75	L4/5	1	LCS	109	29	18	4.20
5	M	70	L2/3/4/5	3	LCS	145	84	24	4.87
6	M	76	L3/4/5	2	LCS	113	150	14	2.56
7	F	56	L4/5	1	LCS	88	2	14	2.72
8	F	71	L4/5	1	LCS	73	13	11	3.46
9	M	72	L5/S1	1	LCS	109	286	12	4.84
10	M	79	L4/5	1	LCS	158	193	20	4.21
11	M	78	L4/5	1	LCS	64	38	23	1.07
12	M	69	L3/4	1	LCS	99	156	17	3.68
13	M	74	L2/3/4	2	LCS	110	25	15	2.05
14	M	73	L2/3/4/5	3	LCS	150	221	17	3.60
15	F	86	L4/5	1	LCS	137	138	27	1.78
16	M	77	L2/3	1	LCS	153	153	11	2.15
17	F	73	L4/5	1	LCS	117	117	17	1.03
18	F	57	L4/5	1	LCS	103	103	10	0.37
19	M	80	L4/5	1	LCS	79	79	18	1.13
20	M	66	L4/5	1	LCS	80	9	18	3.80
21	M	69	L3/4/5	2	LCS	116	82	21	5.24
22	M	65	L3/4/5	2	LCS	150	95	16	3.66

Abbreviations: F, female; LCS, lumbar canal stenosis; M, male; OP, operation.

Assay Kit (Invitrogen, Life technologies; cat #Q32851, lot #1724782). As per the manufacturer's instructions, a standard curve was prepared using the 0 and 10 ng/μL Qubit standards provided in the kit. For all cfDNA extractions, 1 μL of the sample was diluted in 199 μL Qubit working solution before measurement.

2.4 | Pain intensity

The intensity of pain was assessed using a three-level NRS.[8] Each patient was asked to grade the actual pain level experienced at present (present pain; $NRS_{Present}$), maximum pain level experienced in the past 4 weeks (maximum pain; NRS_{Max}), and average pain level experienced in the past 4 weeks (average pain; NRS_{Ave}) on a scale of 0 to 10 (where 0 is no pain and 10 is the worst pain imaginable). NRS scores were obtained for LBP (NRS_{LBP}), LP (NRS_{LP}), and LN (NRS_{LN}). Spearman's correlational analysis assessed the relationships between the patients' cfDNA levels and their respective NRS scores.

2.5 | Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (version 23.0; IBM Corp., Armonk, New York). All values are expressed as mean ± SD. The Shapiro-Wilk test was used to confirm the normality of the data distribution. For the primary analysis, Student *t* test or the Mann-Whitney *U* test was used to compare the two groups. The Student *t* test was used to analyze normally distributed data and the Mann-Whitney *U* test for nonnormally distributed data. The Mann-Whitney *U* test was used to compare cfDNA fragment size, and Student *t* test was used to compare cfDNA levels between control and patient samples. Pearson's product-moment correlation analysis or Spearman's product-moment correlational analysis was used to identifying significant associations.

We used the G-Power Analysis software program to determine sample size validity (G*Power 3.1). Post-hoc analysis using G*Power 3.1 was performed to detect the correlation of subjects and the difference between two independent groups.

The type 1 error was set at 5% for all statistical analyses, and $P < .05$ was significant.

3 | RESULTS

3.1 | Patients characteristics and clinical data

The baseline characteristics and clinical data of the patient and healthy control groups are summarized in Table 2. The pain intensity

scores for the 22 patients were 6.5 ± 2.3 for present pain level experienced at present (present pain; $NRS_{Present}$), 7.7 ± 2.2 for maximum pain level experienced in the past 4 weeks (maximum pain; NRS_{Max}), and 6.7 ± 2.2 for average pain level experienced in the past 4 weeks (average pain; NRS_{Ave}). All patients reported LBP (mean NRS_{LBP} 6.6 ± 2.4), LP (mean NRS_{LP} 6.6 ± 2.6), and LN (mean NRS_{LN} 7.0 ± 1.9). The mean preoperative CRP was 0.30 mg/L (range 0.09-1.76 mg/L) (data not shown).

The operative levels were as follows: L2-3 for 4 patients, L3-4 for 9 patients, L4-5 for 18 patients, and L5-S1 for 1 patient. The mean operative time was 119.9 ± 47.7 minutes (range 64-293 minutes). The mean estimated blood loss was 119.0 ± 149.5 mL (range 2-695 mL). The mean length of hospital stay was 17.5 ± 4.4 days (range 10-27 days). The mean postoperative CRP concentration was 3.02 mg/L (range 0.37-5.24 mg/L).

3.2 | Measurements of cfDNA Fragment Size and cfDNA Level

cfDNA fragment size and cfDNA levels were measured in plasma samples from patients and healthy controls (Figures 1 and 2). The cfDNA fragment size tended to be shorter in patients (mean 166.0 ± 12.8 bp,

TABLE 2 Age and sex distribution of patient cohorts

Patient cohorts		Lumbar canal stenosis (LCS)	Healthy controls (HC)
n		22	5
Age (years)		71.3 (8.1)	38.0 (7.8)
Gender	Male (%)	15 (68.2)	4 (80.0)
	Female (%)	7(31.8)	1 (20.0)
$NRS_{Present}$		6.5 (2.3)	0
NRS_{Max}		7.7 (2.2)	0
NRS_{Ave}		6.7 (2.2)	0
NRS_{LBP}		6.6 (2.4)	0
NRS_{LP}		6.6 (2.6)	0
NRS_{LN}		7.0 (1.9)	0

Note: Data are expressed as mean ± SD.
Abbreviation: NRS, numeric rating scale.

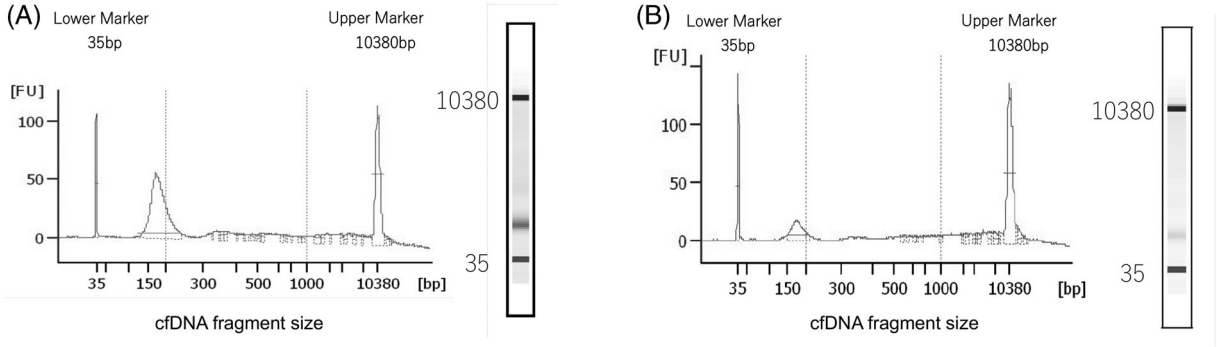

FIGURE 1 Distribution of cfDNA fragment size and cfDNA level in plasma. cfDNA from LCS patients (A) showed larger fragment size (*x*-axis) and cfDNA levels compared to healthy controls (B). 35 bp and 10 380 bp are markers. LCS, lumbar canal stenosis; FU, fluorescence intensity

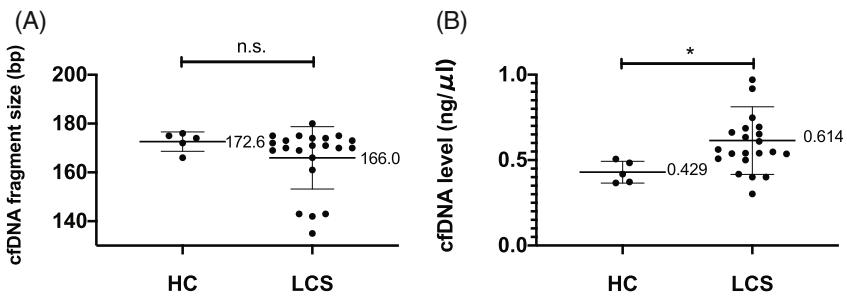

FIGURE 2 Distribution of cfDNA fragment size and cfDNA level in LCS patient and control samples. Each point shows differences in (A) cfDNA fragment size and (B) cfDNA level between samples from healthy controls (HC; n = 5) and patients with a lumbar canal stenosis (LCS; n = 22). HC; healthy controls, LCS, lumbar canal stenosis. n.s., not significant; *$P < 0.05$

range 135-180 bp) than in healthy controls (mean 172.6 ± 4.0 bp, range 166-176 bp), but this difference was not significant ($P = .186$). Plasma cfDNA level was significantly higher in LCS patients (mean 0.614 ± 0.198 ng/μL, range 0.302-1.150 ng/μL) than in healthy controls (mean 0.429 ± 0.064 ng/μL, range 0.366-0.506 ng/μL) ($P = .008$, effect size $d = 1.381$, Power [1 − β error prob] = .764).

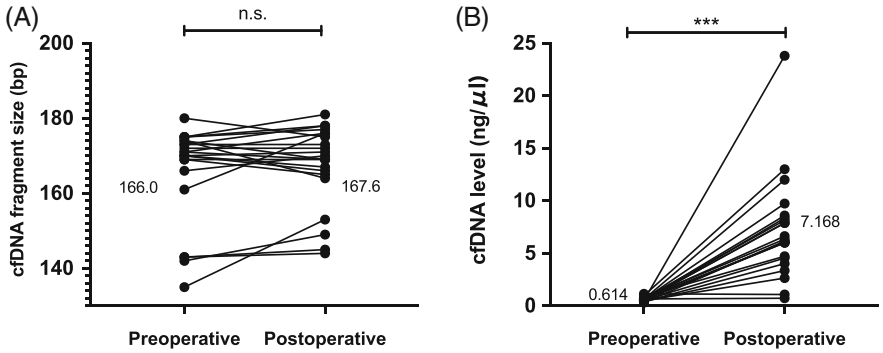

FIGURE 3 Changes in cfDNA fragment size and cfDNA levels from before to after surgery. Each point indicates (A) cfDNA fragment size and (B) cfDNA level before and after surgery for patients with an LCS (n = 22). The data are expressed as mean values. LCS, lumbar canal stenosis. n.s., not significant; ***$P < 0.001$

TABLE 3 Association of cfDNA and each factor

(A) Preoperative cfDNA

N = 27	Preop cfDNA level	Preop cfDNA size	Preop $NRS_{Present}$	Preop NRS_{Max}	Preop NRS_{Ave}	Preop NRS_{LBP}	Preop NRS_{LP}	Preop NRS_{LN}
Preop cfDNA level	1.000							
Preop cfDNA size	0.080	1.000						
Preop $NRS_{Present}$	0.230	0.058	1.000					
Preop NRS_{Max}	0.266	−0.036	0.815***	1.000				
Preop NRS_{Ave}	0.435*	0.065	0.835***	0.893***	1.000			
Preop NRS_{LBP}	0.342	0.107	0.805***	0.710**	0.778***	1.000		
Preop NRS_{LP}	0.369	0.132	0.879***	0.762***	0.807***	0.756***	1.000	
Preop NRS_{LN}	0.451*	0.092	0.721***	0.534**	0.680***	0.693***	0.791***	1.000

(B) Postoperative cfDNA

N = 22	Postop cfDNA level	Postop cfDNA size	Average OR time	Average blood loss	Average length of stay	First-post-OP- CRP	Age	Gender
Postop cfDNA level	1.000							
Postop cfDNA size	−0.237	1.000						
Average OR time	0.348	0.019	1.000					
Average blood loss	0.413	−0.183	0.550**	1.000				
Average length of stay	0.191	0.211	0.142	0.185	1.000			
First-post-OP-CRP	0.269	0.127	0.130	0.236	0.214	1.000		
Age	−0.074	−0.001	−0.052	0.119	0.245	−0.028	1.000	
Gender (0 = F, 1 = M)	−0.023	−0.169	0.131	−0.008	0.116	0.269	0.115	1.000

Note: Spearman's correlation coefficient (*r*) was used to identify significant associations between cfDNA and each factor. *$P < 0.05$, **<0.01, ***<0.001 indicates significant differences.

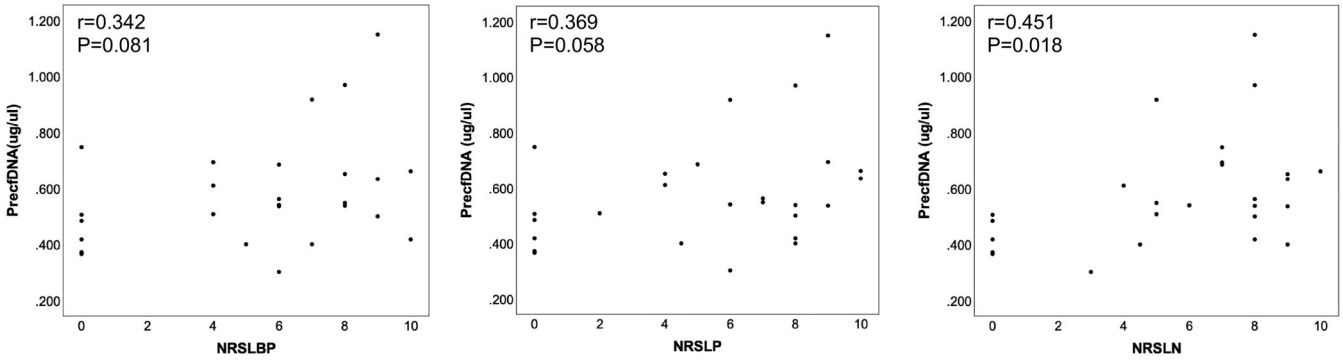

FIGURE 4 Scatter plot and Spearman's correlation between each pain and cfDNA level. Each point on the scatter plot represents one patient. NRS, Numeric Rating Scale; NRSLBP, NRS of low back pain; NRSLP, NRS of leg pain; NRSLN, NRS of leg numbness

3.3 | Changes in cfDNA fragment size and cfDNA levels from before to after Surgery

cfDNA fragment size and cfDNA levels were measured in plasma samples obtained from patients before and after surgery. The median cfDNA fragment did not change from before (mean 166.0 ± 12.8 bp, range 135-180 bp) to after surgery (mean 167.6 ± 10.7 bp, range 144-181 bp) ($P = .242$). By contrast, cfDNA level increased significantly from before (mean 0.614 ± 0.198 ng/μL, range 0.302-1.150 ng/μL) to after surgery (mean 7.168 ± 4.823 ng/μL, range 0.72-23.8 ng/μL) ($P < .001$, effect size $d = 1.920$, Power [1 − β error prob] = .999) (Figure 3).

3.4 | Correlational analysis between cfDNA levels and related factors

A power analysis performed to detect the correlation (effect size $d = 0.5$, alpha = 0.05, total sample size = 27, two-tailed) showed 0.822. We firstly performed correlational analysis between age and cfDNA levels but found no significant correlation between age and cfDNA levels ($r = .326$, $P = .097$, data not shown). Table 3 shows the correlations between cfDNA level or cfDNA fragment size and various indictors of pain and between postoperative cfDNA level and related factors. cfDNA level (n = 27) correlated significantly with NRS_{Ave} ($r = .435$, $P = .026$) and NRS_{LN} ($r = .451$, $P = .018$). Figure 4 demonstrates the distribution of each NRS and cfDNA level. cfDNA fragment size did not correlate significantly with any of these indicators of pain.

Postoperative cfDNA level did not correlate significantly with other factors such as operative time ($r = .348$, $P = .112$), blood loss ($r = .413$ $P = .056$), length of hospital stay ($r = .191$, $P = .394$), postoperative CRP concentration ($r = .269$, $P = .225$), age ($r = -.074$, $P = .743$), and gender ($r = -0.023$, $P = .919$).

4 | DISCUSSION

This is the first study investigating whether cfDNA fragment size and level in LCS patients are associated with various aspects of pain, such as LBP. Elevated cfDNA levels have been reported to be associated with a variety of pathological processes.[9-11] For example, an association between cfDNA and pain has been reported in patients with sickle-cell disease.[12] That study reported that cfDNA levels in patients' plasma were higher during acute painful episodes with steady-state levels.

Against this background, we speculated that the cfDNA fragment size and levels in LCS patients might vary depending on the degree of pain. We first compared cfDNA fragment size and levels in LCS patients and healthy controls. cfDNA fragment size tended to be shorter in patients than in controls, but this difference was not significant. Previous reports on cfDNA in cancer patients showed that cfDNA size is shorter in fragments originating in tumor cells than those from nonmalignant cells.[13-15] An increased number of short cfDNA fragments may reflect an increase in tumor DNA level relative to nontumor DNA in the blood of cancer patients. It is thought that DNA methylation may affect cfDNA size.[16,17] However, the mechanism responsible for the shorter cfDNA fragment size is not well understood.

We also found that cfDNA level was higher in patients with LCS than in healthy controls. Interestingly, the cfDNA level correlated positively with some NRS scores. It is difficult to treat LN in patients with LCS, and postoperative symptoms may remain, and the lack of an index exacerbates this problem to evaluate pain objectively. Our results suggest that the cfDNA level may help quantify leg numbness for patients with LCS.

Subsequently, we investigated whether spinal surgery's invasiveness affects cfDNA fragment size and level by measuring these before and after surgery in LCS patients. We found no significant change in cfDNA fragment size from before to after surgery, although cfDNA level increased postoperatively in all patients. A previous study reported that lactate dehydrogenase (LDH), an enzyme found in various living cells, functions as a biomarker of injury and disease.[18] That study found that elevated cfDNA levels and elevated LDH levels were associated with tissue damage directly associated with pain.[19] Patients were receiving chemotherapy exhibited significant increases in cfDNA levels 24 hours and 8 days after chemotherapy[20] and several cycles after chemotherapy.[21] These data led us to investigate

whether the invasiveness of spinal surgery would be related to an increase in cfDNA levels. In recent years, minimally invasive spinal surgery has attracted much attention. However, surgical time, bleeding loss, length of hospital stay, and postoperative CRP levels are often used to evaluate surgical invasiveness.[22] Surgical procedures induce a complex stress response proportional to the magnitude of the injury, operating time, and intraoperative blood loss. The adverse metabolic and hemodynamic effects of this stress response can cause many problems during the perioperative period. Thus, decreasing the stress response to surgery is a key factor for improving clinical outcomes.

Circulating cfDNA in the blood may be helpful for objectively assessing the body's response to an invasive procedure such as surgery, and its measurement may have potential value for diagnosis and prognosis. Our results suggest that cfDNA level may be a new biomarker for assessing the invasiveness in spinal surgery. A previous report noted that the extent to which postoperative CRP level is elevated seems to depend upon the severity of the procedure.[23] However, there was no correlation between cfDNA level and postoperative CRP level. Further research is needed to clarify whether cfDNA level is related to CRP level and postoperative pain.

A limitation of this study is the small sample size in both the patient and control groups. However, the post hoc power analysis at the cfDNA level was 0.8 or higher between LCS patients and the healthy controls. In addition, the ages of the two groups differed significantly. However, it is difficult to obtain samples from patients aged 70 who do not experience pain because many older people have locomotor disorders. Another limitation is that we did not compare cfDNA fragment size and levels for different surgical procedures. To evaluate surgical invasiveness more concretely, we need to compare cfDNA size and level between decompression surgery and fusion surgery or traditional open surgery and minimally invasive spinal surgery. Additionally, comorbidities can affect the cfDNA fragment size and level; to reduce the possible bias. It would help control for comorbidities in future studies. Finally, we have not investigated the role of cfDNA in predicting symptom response to treatment and sustainability in the long term.

In summary, cfDNA level was higher in patients with LCS than in healthy controls. cfDNA levels increased significantly after spinal surgery and correlated positively with various pains, including average pain or leg numbness. However, it is not possible to conclude whether the observed differences in cfDNA levels are due to age, spinal stenosis, or LBP in only this study. The clinical relevance of an elevated cfDNA level after spinal surgery is unknown, and the utility of cfDNA as a marker of mechanical tissue injury remains unproven. Furthermore, there is still more to be solved, such as where the cfDNA of LCS patients came from and whether it has any function in the event of pain or surgical invasiveness. Further studies involving serial measurements in more significant, independent patient populations are needed to determine whether cfDNA quantification is appropriate for clinical use.

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CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

A.H. participated in the design of the study, performed experiments, analyzed data, and wrote the paper. D.S. participated in the design, and performed experiments, and performed the statistical analysis. S.N. participated in the design. H.K. participated in the design. M.W. participated in the design and coordination. All authors read and approved the final manuscript.

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