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Circulating cell-free DNA fragment analysis by microchip electrophoresis and its relationship with DNase I in cardiac diseases

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ABSTRACT

Circulating cell-free DNA (cfDNA) has been directly related to cancer, diabetes, stroke, systemic lupus erythematosus, trauma, rheumatoid arthritis, inflammation, infection, and myocardial infarction (MI). In this study, plasma cfDNA was extracted from the plasma of cardiac disease patients and the cfDNA fragment distribution as well as the relationships between cfDNA concentration and deoxyribonuclease I (DNase I) activity enzyme implicated in double-stranded DNA processing were examined. Results revealed that the cfDNA concentrations in patients with MI and cardiac angina were significantly higher than that in healthy control subjects. Microchip electrophoresis of plasma cfDNA revealed a single fragment (150–200 bp) in some healthy control subjects and three fragments (150–200 bp, 300–400 bp, and 500–600 bp) in all cardiac patient samples. Moreover, a cfDNA ratio of 150–200 bp/500–600 bp was significantly more prevalent in MI patients than in patients with other cardiac diseases (chest pain, cardiac angina, atrial fibrillation and cardiac failure). In addition, a positive correlation between DNase I activity and cfDNA concentration was observed. These results suggest that the plasma cfDNA in cardiac disease patients may originate from apoptosis and that the 150–200 bp/500–600 bp ratio for cfDNA may be a novel diagnostic indicator for MI.

1. Introduction

Circulating cell-free DNA (cfDNA) was first reported by Mandel and Metais who identified free DNA and RNA in blood plasma [1]. Several reports have suggested that cfDNA originates from the apoptosis or necrosis of all cell types [2–4]. However, apoptosis appears to be the most frequent event that determines the quantity of cfDNA. Alternatively, cfDNA that is released from tumor cells, which is called circulating tumor-cells DNA (ctDNA), is the result of both apoptosis and necrosis [5]. In 1994, the presence of a mutated oncogene product (K-ras) was found in the plasma of pancreatic cancer patients [5]. Several studies have focused on employing ctDNA as a "liquid biopsy" serving as a potential surrogate for tumor biopsies and providing access to the entire tumor genome, thereby enabling early diagnosis with minimal invasion [4–7].

In addition to cancer, cfDNA has been directly related to diabetes, stroke, systemic lupus erythematosus, trauma, rheumatoid arthritis, inflammation, and infection [5]. Chang et al. reported an elevated plasma cfDNA concentration in myocardial infarction (MI) patients when compared to controls [8]. To our knowledge, studies examining fragment analysis of plasma cfDNA in cardiac disease patients are limited [8] and no reports are available on fragment analysis of cfDNA using microchip electrophoresis. Kawai et al. suggested that the serum deoxyribonuclease I (DNase I, EC 3.1.21.1) activity may be applicable as a new diagnostic marker for the early detection of acute MI (AMI) [9]. DNase I is an endonuclease that attacks double-stranded DNA. This enzyme requires divalent cations (Ca^{2+} and Mg^{2+}), is active at neutral pH, produces oligonucleotides with 5′-phospho and 3′-hydroxy termini, and expressed in the pancreas and parotid glands [10]. DNase I has been suggested to be involved in internucleosomal DNA degradation during apoptosis [11], and thus, DNase I elevation in AMI patients may be related to apoptosis induced by ischemic injury [9]. Previous studies reported that DNase I efficiently degrades cfDNA in cancer [12] and it may be involved in the cfDNA elimination system [13]. DNase I is the

Abbreviations: cfDNA, cell-free DNA; MI, myocardial infarction; DNase I, deoxyribonuclease I

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Table 1
Baseline characteristics, plasma cfDNA concentration, and plasma DNase I activity in cardiac disease patients and controls.

	Controls	Chest pain	Myocardial infarction	Cardiac angina	Atrial fibrillation	Takotsubo cardiomyopathy	Cardiac failure
	(n = 62)	(n = 7)	(n = 22)	(n = 15)	(n = 5)	(n = 2)	(n = 6)
Age	34.0 (25–43)	73.5 (65–81)	74.5 (59–80)	76.0 (64–80)	72.5 (58–80)	56.0 –	69.0 (30–72)
Male/female	62/0	4/3	16/6	9/6	2/3	0/2	1/2
cfDNA concentration (μg/mL)	2.60 (1.57–4.02)	15.3 (11.9–18.1)	21.1 (12.4–46.9)	11.1 (6.60–39.1)	8.33 (7.60–23.3)	14.4 –	13.9 (7.65–36.5)
DNase I activity in plasma (units/L)	– –	15.0 (9.16–11.2)	11.4 (9.67–17.6)	15.6 (10.0–23.6)	13.0 (10.6–19.1)	9.57 –	10.5 (6.22–11.0)

Values are median (interquartile range).

primary factor responsible for regulating the elimination and degradation of cfDNA from the blood stream [12,13]. Currently, there are only two studies that have investigated the relationship between cfDNA concentration and DNase I activity [14,15]. Therefore, the aim of this study was to perform fragment analysis on plasma cfDNA from cardiac disease patients using microchip electrophoresis and to investigate the relationships between DNase I activity and cfDNA concentration.

2. Materials and methods

2.1. Study population

Blood samples were collected from cardiac disease (chest pain, MI, cardiac angina, atrial fibrillation, Takotsubo cardiomyopathy, cardiac failure) patients (n = 57) who had presented at emergency outpatient services at Shimane University Hospital (Shimane, Japan) from 2016 to 2018 (Table 1). No cancer patients were enrolled in the study. Chest pain patients were ruled out as without AMI or unstable angina by coronary angiography.Control blood samples were collected from 62 healthy Japanese volunteers living in Shimane Prefecture. At the time of emergency room admission, venous blood was drawn and placed into tubes containing heparin, and the plasma was separated by centrifugation at 500 xg and stored at −70 °C.

The study including usage of plasma and DNA derived from patients and control subjects was reviewed and approved by the Human Ethics Committee of Shimane University School of Medicine. In addition, informed consent was obtained from all participants.

2.2. Cell-free DNA isolation from plasma

Previous study demonstrated that the yield of cfDNA extraction and even the profile of cfDNA fragments recovered are method-dependent [16].They showed that cfDNA yield by the Maxwell® RSC ccfDNA Plasma Kit was higher than that by the QIAamp Circulating Nucleid Acid kit and the MagNA Pure Compact Nucleic Acid Isolation kit and that the profile of cfDNA fragments recovered by the Maxwell® RSC ccfDNA Plasma Kit were lower in mono-nucleosomes fragments, higher in di-and ti-nucleosomes than by the MagNA Pure Compact Nucleic Acid Isolation kit. Therefore, in the present study, the Maxwell® RSC ccfDNA Plasma Kit (Promega Corp., Madison, WI, USA) was used with the Maxwell® RSC Instrument to purify cfDNA from 1 ml plasma.

The cfDNA concentrations were measured with a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Two microliters of each sample was plated on Thermo Scientific™ μDrop™ plates and spectrophotometric absorbances at 260 nm, 280 nm, and 320 nm were measured.

2.3. Microchip electrophoresis

Microchip electrophoresis is fast and sensitive, results in high resolution, and allows for the easy separation of DNA. Furthermore, it is one of the most successful miniaturized analysis systems, promising minimal sample and reagent consumption, short analysis time, efficiency, integration, and automation [17].

Extracted cfDNA was visualized using MCE-202 MultiNA automated microchip-based electrophoresis equipped with MultiNA Viewer software (Shimadzu Corp., Kyoto, Japan). MCE-202 MultiNA utilizes lab-on a-chip technology to perform gel electrophoresis. It can perform high-throughput (up to 96 samples), high-speed and high reproducible sample size confirmation using cost-effective reusable microchip. Samples were run with the reagents from the DNA-1000 kit and DNA-2500 kit (Shimadzu), namely separation buffer and DNA marker reagent. A 100 bp DNA ladder (1000 kit, Takara Bio, Inc., Shiga, Japan) and pGEM DNA marker (2500 kit, Promega corp., Madison, USA) were employed as a ladder marker.

2.4. DNase I activity assay

DNase I activity in plasma was assayed using the single radial enzyme diffusion (SRED) method [18] according to our previous study [19]. Briefly, a 50 mM sodium cacodylate buffer (pH 6.5) containing 10 mM MgCl2 and 1 mM CaCl2 was used as the reaction buffer. Eleven milliliters of a reaction buffer was combined with 350 μl of 1% salmon testis DNA and 35 μ l of 1% ethidium bromide and vortexed. To this, 11 ml of 2% molten agarose GP-36 was added, and the mixture was immediately poured onto a horizontal Agafix MSL sheet (7.5 × 13.5 cm) (Wako Pure Chemical Industries, Tokyo, Japan). Following congelation at room temperature, rows of cylindrical sample wells (radius [r0] = 1.0 mm) with centers 15 mm apart were punched into the gel. Samples (5 μl) were then added to the wells and incubated in a moist chamber at 37 °C for 20 h. DNase I activity was observed under UV (312 nm). The radius of each dark circle in each well was measured, and the DNase I activity was calculated by comparing with the standard curve.

2.5. Statistical analysis

The differences in cfDNA concentration between control groups and patient groups were analyzed by performing Dunnett's test. Differences within each patient group were analyzed with Scheffe's test. Single regression analysis was performed to estimate the relationship between DNase I activity in plasma and plasma cfDNA concentration. These statistics were conducted using the program STATCEL2 (OMS Publishing, Inc. Tokorozawa, Saitama, Japan).

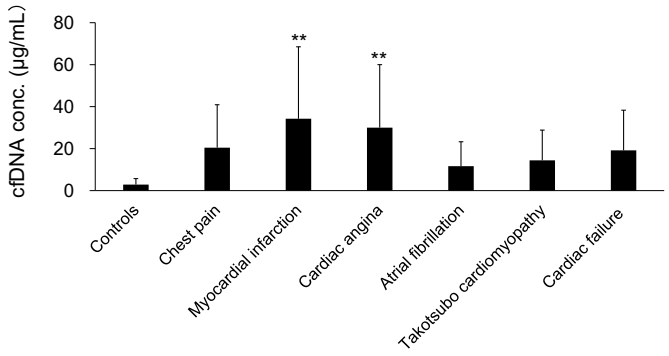

Fig. 1. The cfDNA concentrations in plasma from cardiac disease patients and healthy control subjects. Data were analyzed with Dunnett's test and are expressed as mean ± S.D. Significantly higher levels of cfDNA were detected in myocardial infarction and cardia angina patients when compared to those in normal controls. $^{**}p < 0.01$ when compared with the control group. Significant differences were not observed between individual disease groups following Scheffe's test analysis.

3. Results and discussion

3.1. cfDNA concentrations

Plasma cfDNA concentration in control subjects (Table 1) were similar to that in control subjects from previous studies [15,20]. Previous studies have reported a > 10-fold elevation of cfDNA in MI patients when compared to normal control individuals [8]. In the present study, cfDNA concentrations in healthy control subjects and various cardiac disease patients were quantified (Fig. 1). Elevated plasma cfDNA concentrations were observed in all cardiac diseases. Significantly higher cfDNA concentrations were observed in MI ($p < 0.01$) and cardiac angina ($p < 0.01$) patients when compared to control subjects (a 15-fold elevation). Significant differences were not observed between individual disease groups, according to Scheffe's test analysis (data not shown). Previous studies have reported that ischemic injuries induce apoptosis in AMI [21,22]. Therefore, the elevated levels of cfDNA in ischemic heart disease (MI and cardiac angina) patients in our study may have been the results of apoptosis.

3.2. Microchip electrophoresis of cfDNA

Plasma cfDNA from healthy control subjects and cardiac disease patients was electrophoresed and representative gel-like images and electropherograms are shown in Fig. 2 and Fig. 3. In healthy control samples, a single band 150–200 bp region was observable in some lanes, but no fragments were observed in some healthy subject (Fig. 2). This single fragment has reported by the previous study which may originates from the cell death of an organ [6]. Alternatively, three fragments (150–200 bp, 300–400 bp, and 500–600 bp) were observed in all MI, cardiac angina patients (Fig. 3) and all other cardiac patient samples (data not shown).

Several previous studies have analyzed the cfDNA fragments from a wide spectrum of patients by using capillary/microchip electrophoresis. Qutinen et al. revealed that the plasma cfDNA in patients with *Puumala hantavirus* infection displayed a low molecular weight appearance, corresponding to the size of apoptotic DNA fragments (150–200 bp) [20]. Additionally, Kwee et al. have shown the cfDNA 160–200 bp fragment, consistent with cfDNA released from apoptotic cells, in plasma from prostate cancer patients [23]. In an additional study, a 166 bp fragment was observed in noncancer controls and 166 bp, 332 bp, and 498 bp fragments were observed in the plasma of cancer patients [6]. It was suggested that these three fragments indicate that apoptosis is the primary driver for the release of DNA fragments into the circulation [6]. Another *in vivo* study using cancer cells revealed that apoptosis produces fragments that are the same size as a typical ladder pattern (150–200 bp, 300–400 bp, and 500–600 bp) and this pattern is characteristic to nucleosomal subunits [24]. Our results suggest that apoptosis occurs in all cardiac disease patients to some extent. To our knowledge, no study is available on plasma cfDNA from cardiac disease patient electrophoresis using capillary/microchip electrophoresis. Chang et al. reported gel electrophoresis patterns of plasma cfDNA for MI patients showing that DNA fragments occur at approximately 500 bp, 300–400 bp, and 200 bp (major band) [8]. This result agrees with our study.

Using a DNA-2500 kit, plasma cfDNA concentrations for each fragment of cardiac disease patients as well as the ratio of each fragment's concentration were evaluated. Results indicate that a 150–200 bp/500–600 bp ratio in MI was significantly higher than those in other cardiac disease (chest pain, cardiac angina, atrial fibrillation and cardiac failure) patients (Fig. 4). Although the sample size is small, this ratio may be relevant for use in differential diagnosis.

The cardiac troponin I (cTnI) with high sensitive method is widely used as biomarker of subtle cardiac damages as apoptosis or reversible cardiomyocyte membrane leakage [25]. cTnI release after brief ischemia was delayed but increase to detectable levels between 3 and 24 h. Previous study have reported that cTnI appeared in blood at 3 to 8 h, peaked at 10–20 h following the onset of AMI. [26]. On the other hand, Bliksøen et al. found that circulating cell-free mtDNA in the serum of AMI patients appeared in blood at 1 h and peaked at 3 h after the onset of AMI [27]. O'Connell et al. reported that the cfDNA levels are acutely elevated in ischaemic stroke [28]. Plasma cfDNA may have advantage for early diagnosis. Due to its advantage, several studies indicate that the cfDNA may serve as a potential diagnostic and prognostic biomarker for AMI [26,27,19,30].

3.3. Relationship between DNase I activity and cfDNA concentration

The relationship observed between DNase I activity and cfDNA concentration in the plasma from all cardiac disease patients is shown in Fig. 5. Regression analysis revealed apositive correlation between DNase I activity and cfDNA concentration ($p < 0.05$; $R = 0.31$). This result is distinct from previous studies. Specifically, Ershova et al. described a negative correlation between cfDNA concentration and DNase I activity in blood of healthy non-pregnant women, thereby suggesting that increased cfDNA concentration caused by cell death leads to activation of a cfDNA eliminating system (DNase I), resulting in decreased cfDNA concentration [14]. Additionally, Golonka et al. suggested that DNase I activity is a superior biomarker for liver cancer compared to cfDNA. They hypothesized that the reason cfDNA may not be considerably elevated in cancer patients is that elevation of cfDNA levels may be regulated by increased DNase I expression [15]. Korzeneva et al. also observed significantly reduced cfDNA concentrations in cases of chronic processes (that are accompanied by increased cell death) [31]. The observed variation between our results and those of other studies may be caused by differences between chronic and acute processes as well as differences that exist between cancer and cardiac disease. Further investigations are required to elucidate the cause for this variation.

cfDNA exerts active biological effects. A previous study reported the alteration of hemodynamic properties of the blood by cfDNA [32]. It can cause oxidative stress and stimulate proinflammatory cytokine synthesis [33]. Mittra et al. reported that the apoptosis-derived cfDNA can damage cells and the effects are different between cfDNA from cancer cells and those from healthy controls [34]. To date, the effects of cfDNA on cardiac diseases remain unknown; however, cfDNA has the potential to affect the pathological conditions associated with cardiac disease. Further studies are, therefore, needed to reveal the mechanism of action of cfDNA on cardiac disease.

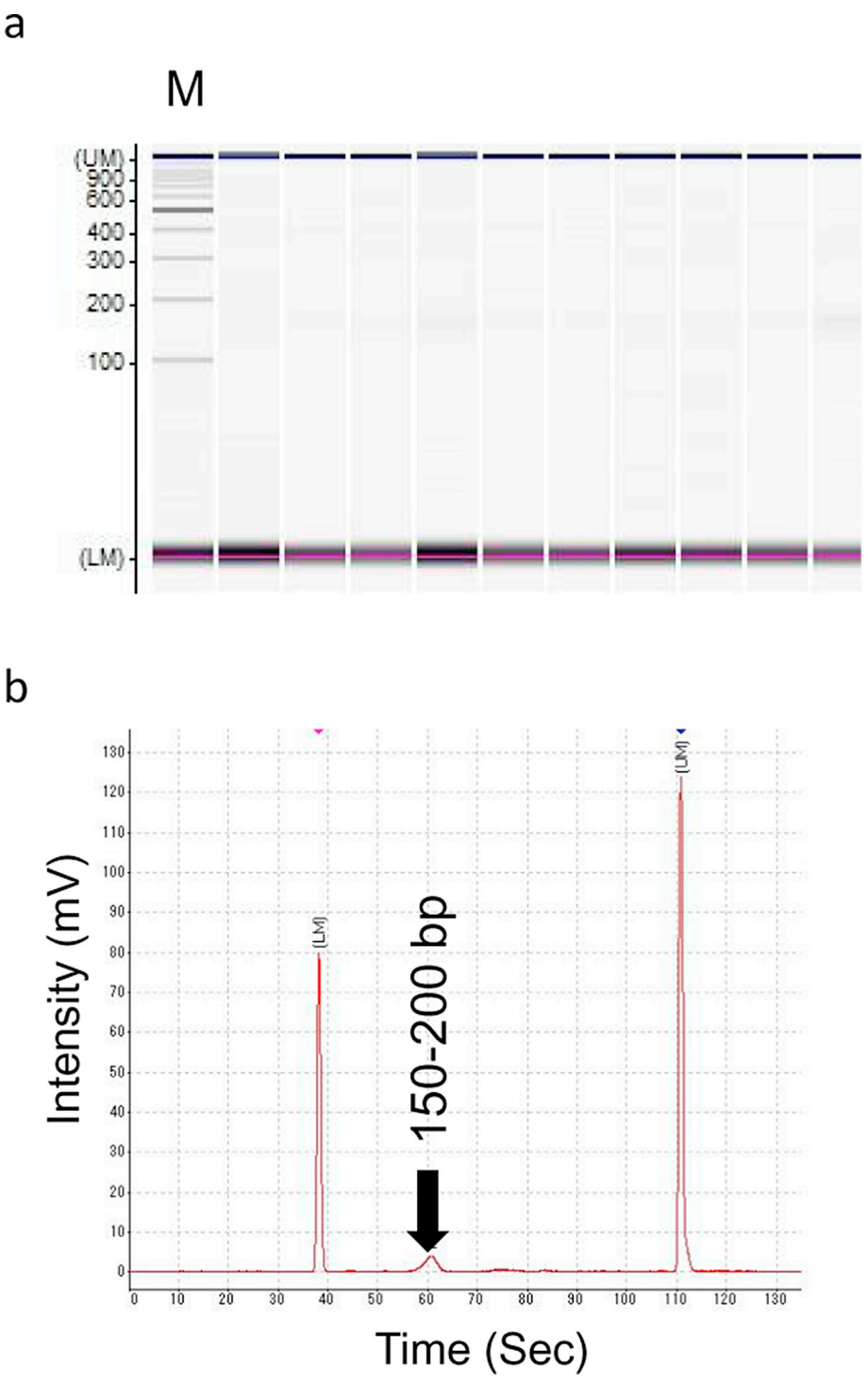

Fig. 2. Representative gel-like image (a) and electropherogram (b) of the cDNA from control subjects. M: 100 bp ladder marker.

4. Conclusion

In the present study, cfDNA was extracted from plasma of cardiac disease patients and investigated for fragment distribution of cfDNA. We also examined the relationships between cfDNA concentration and DNase I activity. The cfDNA concentrations from patients with MI, cardiac angina, were significantly higher than that of healthy control subjects. Microchip electrophoresis of plasma cfDNA revealed a single fragment (150–200 bp) in healthy control subjects while ladder pattern (150–200 bp, 300–400 bp, and 500–600 bp), which is characteristic to nucleosomal subunits, were identified in all cardiac patient samples. In addition, a positive correlation was identified between DNase I activity and cfDNA concentration. These results suggest that the plasma cfDNA in cardiac disease patient may originate from apoptosis. In addition, the 150–200 bp/500–600 bp ratio in MI patient samples was significantly higher than those observed in other cardiac disease patients. Preliminary experiments suggest that the 150–200 bp/500–600 bp concentration ratio in plasma cfDNA from MI patients may be effective as a diagnostic parameter.

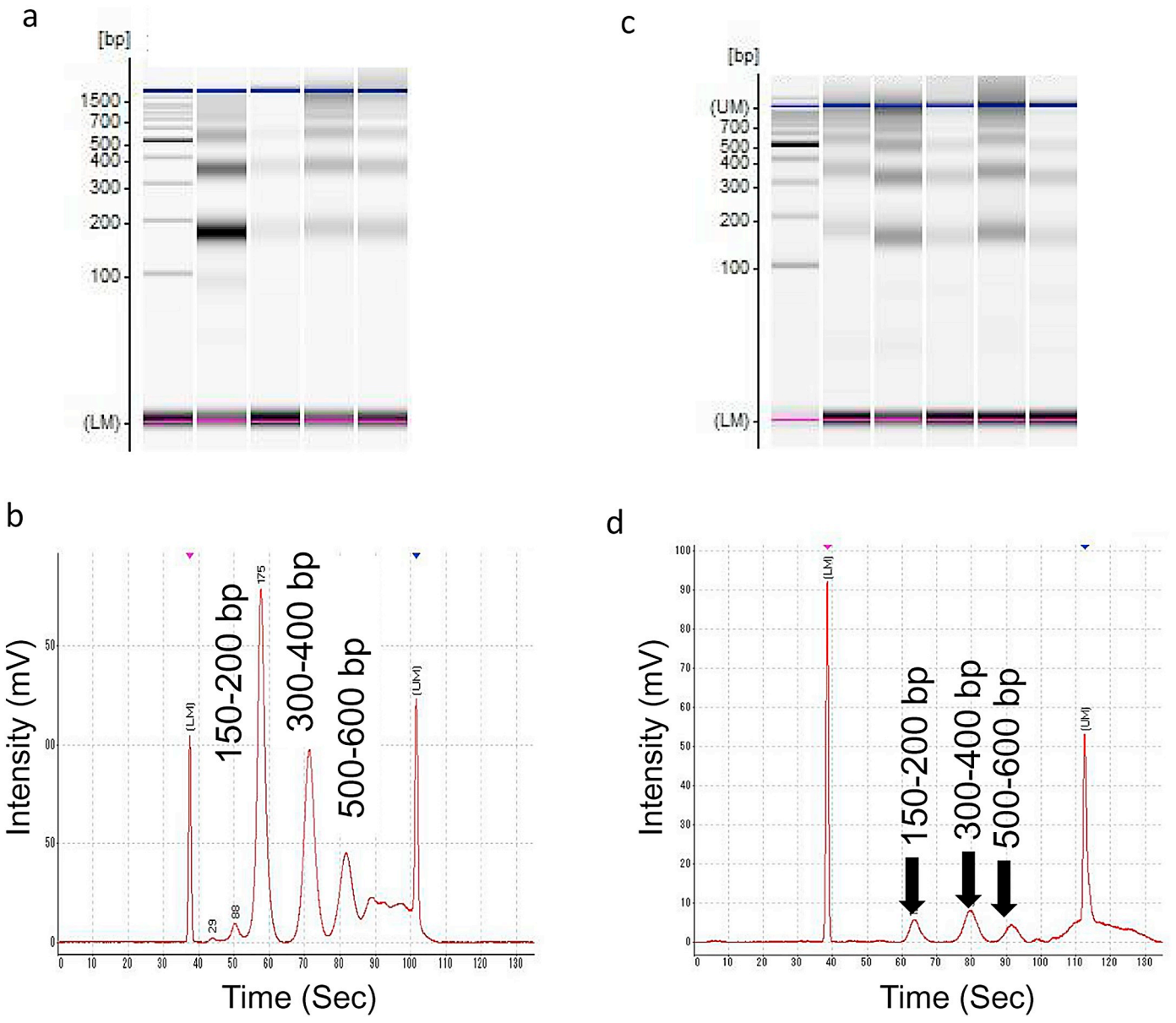

Fig. 3. Representative gel-like image and electropherogram of the cfDNA from myocardial infarction (a, b) and cardiac angina(c, d) patients. M: 100 bp ladder marker.

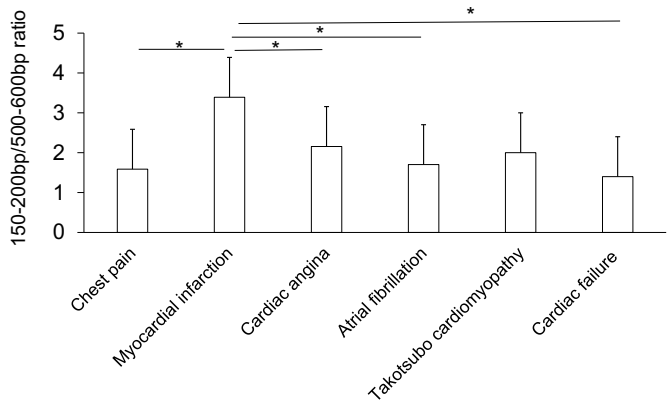

Fig. 4. The 150–200 bp/500–600 bp cfDNA fragment concentrations ratio from plasma of cardiac disease patients. Data are expressed as mean ± S.D. $^{*}p < 0.05$ when compared using Scheffe's test. CfDNA levels of myocardial infarction were significantly higher than those of chest pain, cardiac angina, atrial fibrillation and cardiac failure.

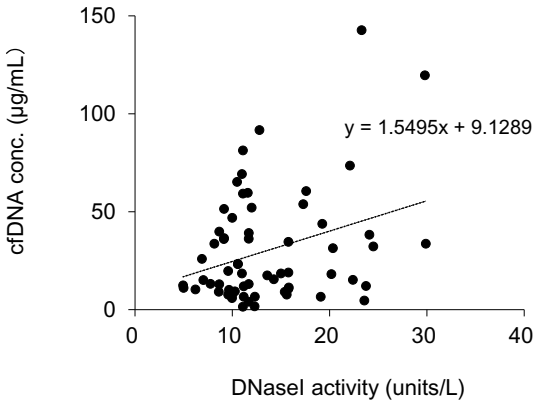

Fig. 5. Relationship between DNase I activity and cfDNA concentration in the plasma of all patients with cardiac diseases.

5. Limitations

Limitation of this study is the small number of patients. Therefore, further confirmation of these data using more patient samples is needed before employing this ratio in the diagnosis of MI.

Declaration of Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgments

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