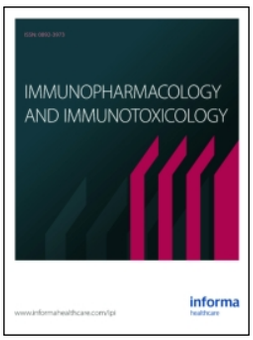

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ORIGINAL ARTICLE



# Integrity of circulating cell-free DNA as a prognostic biomarker for vaccine therapy in patients with nonsmall cell lung cancer

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**ABSTRACT**

**Background:** Many clinical trials of immune checkpoint blockade-based combination therapies are under way. Vaccine therapy is a promising partner of combination therapies. We have developed a personalized peptide vaccination and conducted clinical trials of it in patients with various cancers. At the present time, we have only a limited number of biomarkers related to the prognosis of vaccine-treated patients. Thus, new biomarkers are urgently needed.

**Methods:** In this study, we investigated the plasma cell-free DNA (cfDNA) integrity—a ratio of the necrotic tumor cell-derived long cfDNA fragments to the total dead cell-derived short cfDNA fragments from genomic Alu elements—in patients with advanced nonsmall cell lung cancer during treatment with the personalized peptide vaccination.

**Results:** We found that (1) the cfDNA integrity was decreased after the first cycle of vaccination, and (2) the patients with high prevaccination cfDNA integrity survived longer than those with low prevaccination integrity (median survival time (MST): 17.9 versus 9.0 months, respectively; hazard ratio (HR): 0.58, $p = .0049$). A similar tendency was observed in postvaccination cfDNA integrity (MST: 16.4 vs 9.4 months; HR: 0.65, $p = .024$).

**Conclusions:** These results suggest that cfDNA integrity is a possible prognostic biomarker in patients treated with the personalized peptide vaccine.

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**KEYWORDS**
DNA integrity; non-small cell lung cancer; peptide vaccine; prognostic biomarker; immunotherapy

## Introduction

Lung cancer is the most common cancer in the world; each year nearly 2.1 million individuals newly develop lung cancer and 1.76 million die from it [1]. About 80–85% of lung cancers are nonsmall cell lung cancers (NSCLCs), consisting predominantly of adenocarcinoma and squamous cell carcinoma (SCC) [2]. Within the last decade, several new molecular-targeted drugs have been developed, such as tyrosine kinase inhibitors (TKIs) targeted to mutated epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) [3]. More recently, immune checkpoint blockades (ICBs), such as monoclonal antibodies against programmed cell death protein 1 (PD-1) and its ligand, programmed death-ligand 1 (PD-L1), have been approved for the treatment of NSCLC [3]. However, the prognosis of advanced NSCLC is still poor, and new therapeutic modalities are thus urgently needed.

Many clinical trials of ICB-based combination therapies are underway [4]. Vaccine therapy is a promising partner of combination therapies [5]. We have developed a personalized peptide vaccination, in which a maximum of four immunocompetent cytotoxic T-lymphocyte (CTL)-epitope peptides were selected from 31 candidate peptides based on each patient's HLA-A locus type and pre-vaccination immunity to the peptides. We have used this vaccine with Montanide ISA51VG as an adjuvant [6,7]. Clinical trials of the vaccine have been conducted in patients with various cancers [6–8]. Results of an early phase II study of the vaccine in patients with NSCLC suggested the vaccine's feasibility for the treatment of refractory NSCLC [9]. A subsequent study also suggested the feasibility of the vaccine for heavily treated advanced NSCLC patients who failed two or more treatment regimens [10]. A randomized controlled phase II study of docetaxel plus the vaccination versus docetaxel plus placebo in patients with previously treated advanced wild-type EGFR NSCLC was also conducted [11]. Although the primary endpoint, i.e. improvement of progression-free survival (PFS), was not reached in that study, the PFS and overall survival (OS) were significantly longer in IgG responders against vaccinated peptides than in nonresponders [11]. These results suggested that the vaccine may prolong the survival of IgG responders. At the present time, we have only a limited number of biomarkers related to the prognosis of vaccine-treated patients. Thus, new biomarkers are urgently needed.

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In this study, we investigated the plasma cell-free DNA (cfDNA) integrity—a ratio of the necrotic tumor cell-derived long cfDNA fragments to the total dead cell-derived short cfDNA fragments from genomic Alu elements—in patients with advanced NSCLC during treatment with the personalized peptide vaccination that we developed.

## Patients and methods

### Plasma samples

The study used frozen plasma samples from 130 patients with advanced NSCLC who were enrolled in clinical trials of the personalized peptide vaccination from January 2009 to July 2012. Patient characteristics and the clinical protocols of the vaccination have been reported [9,10]. The clinical study was approved by the Kurume University Ethics Committee and registered with the UMIN Clinical Trial Registry under trial numbers UMIN1839 and 2984. Plasma samples obtained before and after the first vaccination cycle, consisting of weekly injection for 6 or 8 weeks, were used.

### cfDNA integrity

The method of analyzing cfDNA integrity has been described elsewhere [12]. In brief, 1:40 diluted unpurified plasma samples were used as cfDNA for the amplification of Alu fragments. Short and long Alu fragments were amplified and quantitated using real-time polymerase chain reaction (PCR) (StepOne plus, Thermo Fisher Scientific, Waltham, MA) with THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan). The PCR primer pairs were as follows: forward, 5′-CCTGAGGTCA GGAGTTCGAG-3′ and reverse, 5′-CCTGAGGTCAGGAGTTCGAG-3′ for Alu-115; forward, 5′-GTGGCTCACGCCTGTAATC-3′ and reverse, 5′-CAGGCTGGAGTGCAGTGG-3′ for Alu-247. Amplification was performed based on 40 cycles at 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s, following the initial denaturation at 95 °C for 10 min. Short (115 bp) and long (247 bp) PCR fragments of Alu reflected total cfDNA and cfDNA derived from necrotic cells (mainly tumor cells), respectively. cfDNA integrity was calculated according to the formula:

$$\text{cfDNA integrity} = 2^{(\text{Ct value of Alu}-115 - \text{Ct value of Alu}-247)}$$

### Measurement of peptide-reactive IgG and CTLs

Vaccinated peptide-reactive IgG in the plasma and CTLs were quantitated as described previously [9,10]. The CTL responses were measured by an ELISPOT assay of interferon-gamma–secreting cells. If the IgG levels or spot number against at least one vaccinated peptide were more than twice the pre-vaccination level, the response was considered augmented.

### Statistical analysis

The survival curves were plotted by the Kaplan–Meier method. We used a Cox hazard model to compare the high and low groups for cfDNA integrity before and after the first cycle of vaccination. cfDNA and integrity levels of pre- and postvaccination were compared by Wilcoxon signed rank test. cfDNA integrity and IgG or CTL responses were compared by Fisher's exact probability test. Statistical analyses were performed using JMP Pro version 13 software (SAS Institute, Cary, NC).

## Results

### Alteration of circulating cfDNA integrity during peptide vaccination

The total cfDNA (Alu-115: the short 115-bp PCR fragment of Alu) and the necrotic cell (mainly tumor cell)-derived cfDNA (Alu-247: the long 247-bp PCR fragment of Alu) of plasma samples from 130 patients with advanced NSCLC were analyzed. Patients' characteristics were as follows: adenocarcinoma ($n = 101$), SCC ($n = 25$), adenosquamous carcinoma ($n = 2$), large cell carcinoma ($n = 1$), and pleomorphic carcinoma ($n = 1$). Patient stages were as follows: stage III ($n = 18$), stage IV ($n = 73$), and recurrent ($n = 39$). Representative PCR amplification curves are shown in Figure 1. Figure 2 shows the total cfDNA (Alu-115), necrotic cell-derived cfDNA (Alu-247), and the cfDNA integrity (Alu-247/Alu-115) of pre- and post-first cycle of vaccination. Decreases in Alu-115 (increase in Ct of Alu-115) and cfDNA integrity during vaccination were observed ($p < .0001$ and $p = .0005$, respectively), although significant alteration of Alu-247 was not observed.

### Relationship between circulating cfDNA integrity and prognosis

The patients were divided into high and low groups of cfDNA integrity at pre- or post-vaccination, and their OS was analyzed by a Kaplan–Meier plot (Figure 3). The median values of cfDNA integrity were used to define 'high' and 'low'.

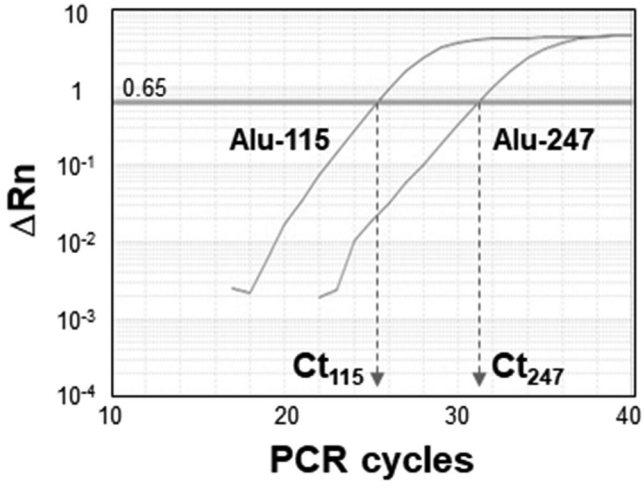


Figure 1. Representative amplification curves for Alu-115 and Alu-247. An arbitrary cut-off value of ΔRn = 0.65 was used to obtain the Ct values. $Ct_{115}$ and $Ct_{247}$ mean Ct values of Alu-115 and Alu-247, respectively.

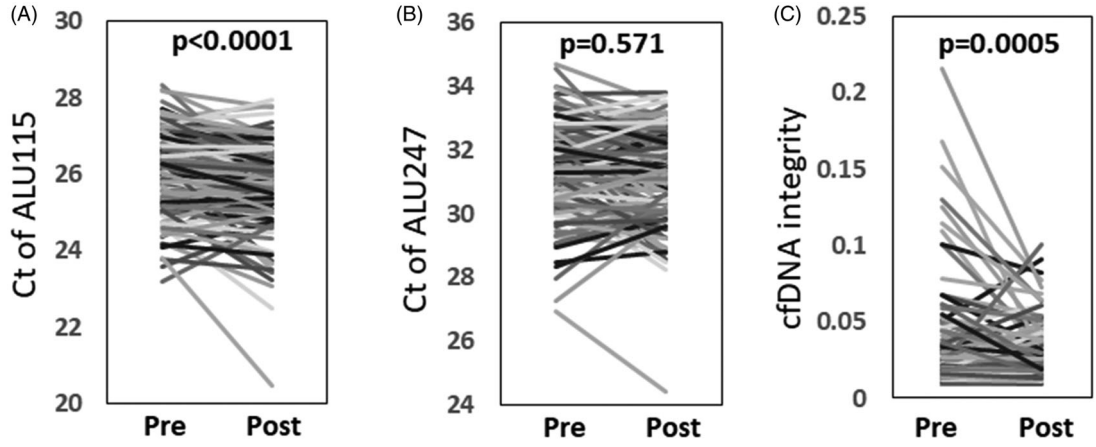


**Figure 2.** Total cfDNA (Alu-115) (A), tumor cell-derived cfDNA (Alu-247) (B), and the cfDNA integrity (Alu-247/Alu-115) (C) of pre- and post-first cycle vaccination are shown ($n = 130$).

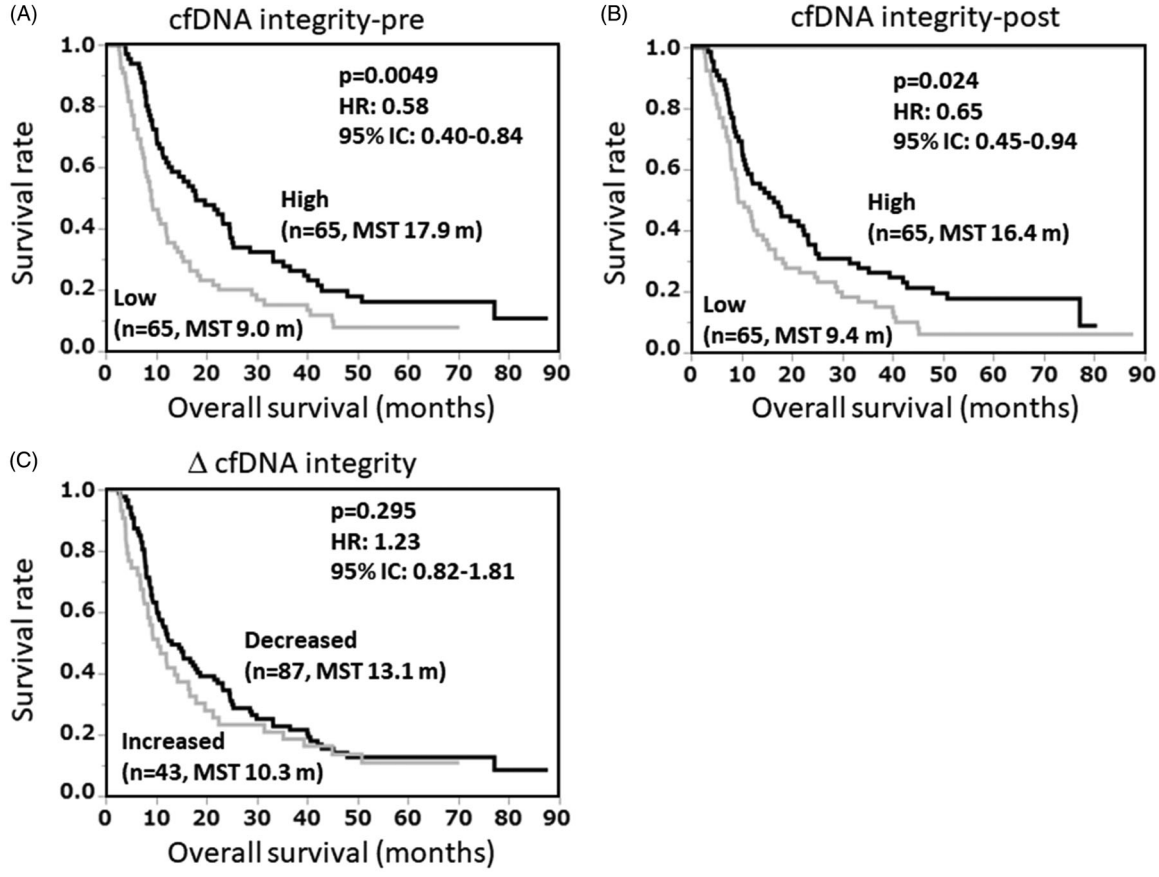


**Figure 3.** The patients ($n = 130$) were divided into high and low groups of cfDNA integrity of pre-vaccination (A), post-first cycle vaccination (B), or the difference in cfDNA integrity between pre- and post-first cycle vaccination (Δ cfDNA integrity) (C) and the overall survival were analyzed by the Kaplan–Meier plot. *P*-values show the results of the Cox hazard model.

Overall survival of the pre-vaccination cfDNA integrity high group (median survival time: MST = 17.9 months, $n = 65$) was significantly longer than that of the low group (MST = 9.0 months, $n = 65$) (HR = 0.58, 95%CI: 0.40-0.84, $p = .0049$). A similar result was observed in the post-vaccination cfDNA integrity high group (MST = 16.4 months, $n = 65$) and low group (MST = 9.4 months, $n = 65$) (HR = 0.65, 95%CI: 0.45-0.95, $p = .024$). The relationship between OS and the difference in cfDNA integrity among the pre- and post-vaccination values (Δ cfDNA integrity) was also analyzed. However, no significant difference was observed between the Δ cfDNA integrity increased and decreased groups.

### *Relationship between cfDNA integrity and vaccine-induced immune responses*

The augmentation of IgG response against at least one vaccinated peptide was detected in 83 (66.2%) of a total of 130 patients after the first cycle of vaccination. Fifty-eight patients were subjected to CTL response analyses, since

sufficient blood samples from pre- and post-vaccination were not available from the remaining patients. The augmentation of CTL response against at least one vaccinated peptide was detected in 26 (44.8%) of a total of 58 patients after the first vaccination cycle. The distribution of the numbers of IgG and/or CTL response-augmented (positive) peptides of the 58 patients is shown in Figure 4.

We subsequently analyzed the relationship between cfDNA integrity and vaccine-induced immune responses. Figure 5 shows the pre- and post-vaccination cfDNA integrity, as well as the alterations in cfDNA integrity (Δ cfDNA integrity), in the IgG response-positive and -negative (=not augmented) groups. Neither the pre- and postvaccination cfDNA integrity, nor the Δ cfDNA integrity, in the IgG response-positive group ($n = 83$) differed significantly from those in the IgG response-negative group ($n = 47$) (Figure 5(A–C)). The proportions of cases in which cfDNA integrity was decreased after vaccination were 59.5 and 71.0% in the IgG response-negative and -positive groups, respectively, with no statistical difference between the two groups (Figure 5(D)). The IgG response-positive group was further divided into four subgroups according to the number of IgG response-positive peptides (a total of five groups including a zero-peptide group), and the difference among the subgroups of the pre- and post-vaccination cfDNA integrity and Δ cfDNA integrity were analyzed. However, no significant difference was found among the subgroups in each factor (Figure 5(E–G)).

Figure 6 shows the pre- and post-vaccination cfDNA integrity, as well as Δ cfDNA integrity in the CTL response-positive ($n = 26$) and -negative ($n = 32$) groups. Similar to the case with IgG response, neither the pre- and post-vaccination cfDNA integrity, nor the alteration of the cfDNA integrity, in the CTL response-positive group differed significantly from those of the CTL response-negative group (Figure 6(A–C)). Decreases in cfDNA integrity during vaccination were observed in 78.1 and 69.2% of the CTL response-negative and -positive groups, respectively, with no statistical difference between the groups (Figure 6(D)). The CTL response-positive group was further divided into two subgroups

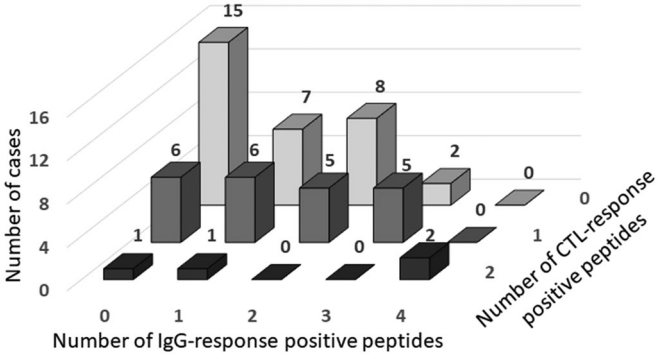


**Figure 4.** Distribution of the numbers of IgG and/or CTL response augmented (positive) peptides of the 58 patients.

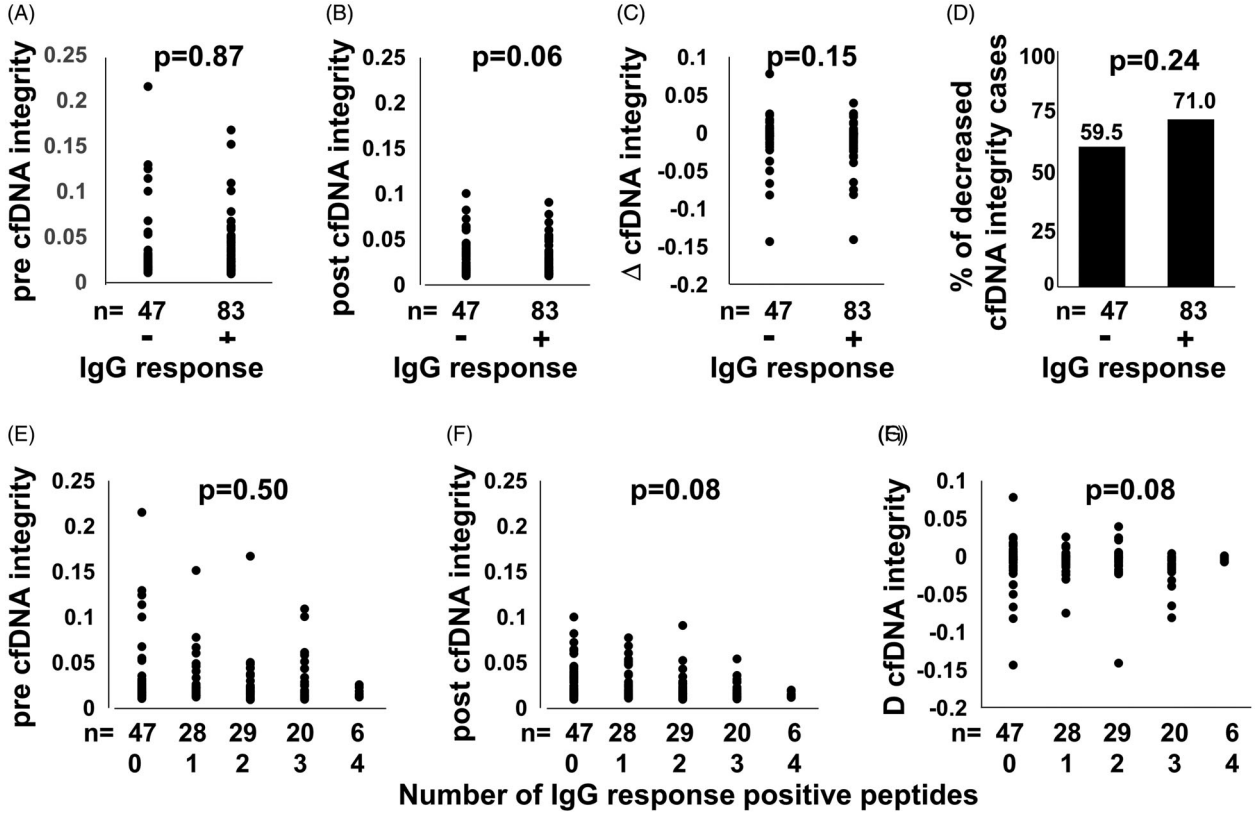


**Figure 5.** Relationship between cfDNA integrity and vaccine-induced IgG response ($n = 130$). Pre- (A, E) and post- (B, F) vaccination cfDNA integrity, and the alterations in the cfDNA integrity (Δ cfDNA integrity) (C, G) of the IgG response-positive and -negative groups (A-C) or of the subgroups of IgG response-positive peptides (E–G) are shown. (D) Percentages of cfDNA integrity-decreased cases in the IgG response-positive and -negative groups are shown. The differences among the IgG response-positive and -negative groups or subgroups in pre- and post-vaccination cfDNA integrity, Δ cfDNA integrity, or the percentages of cfDNA integrity-decreased cases were analyzed using the nonparametric Wilcoxon test.

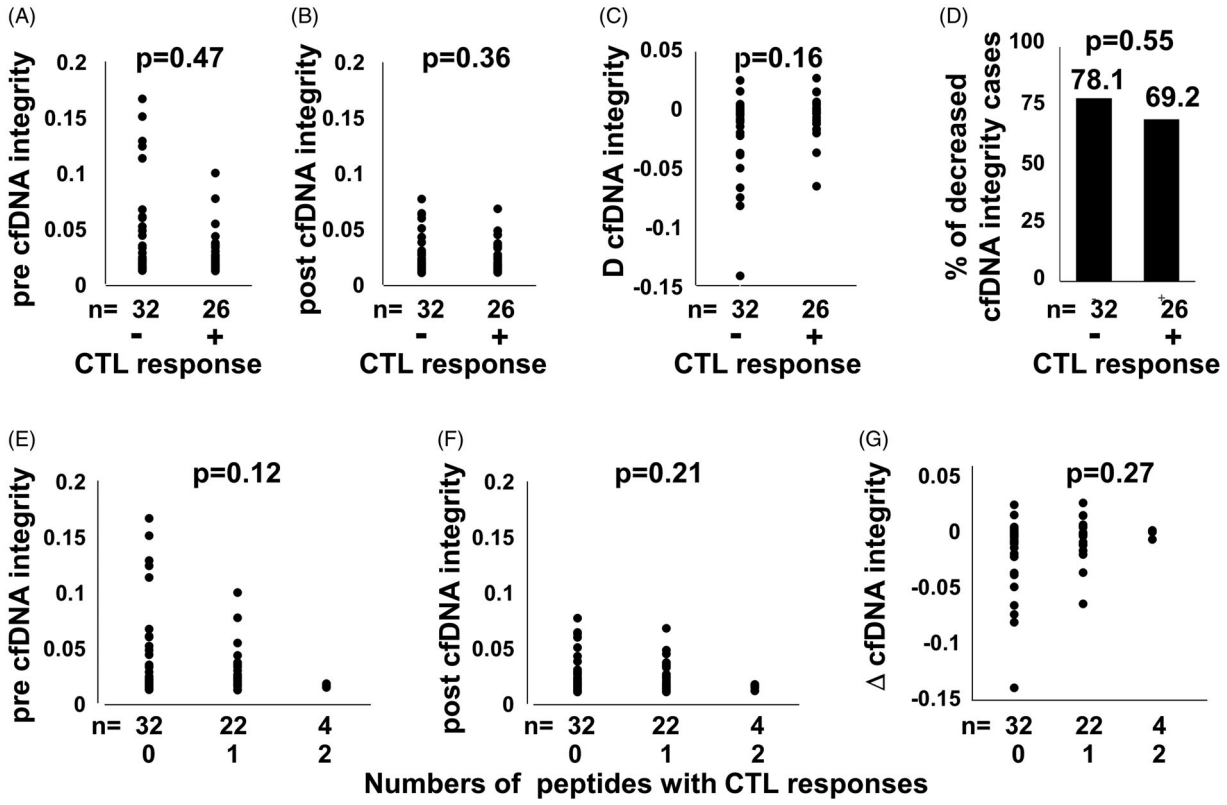


**Figure 6.** Relationship between cfDNA integrity and vaccine-induced CTL response ($n = 58$). Pre- (A, E) and post- (B, F) vaccination cfDNA integrity, and the alterations of cfDNA integrity (Δ cfDNA integrity) (C, G) of the CTL response-positive and -negative groups (A–C) or of the subgroups of the number of CTL response-positive peptides (E–G) are shown. (D) Percentages of cfDNA integrity-decreased cases in the CTL response-positive and -negative groups are shown. The difference among the CTL response-positive and -negative groups or subgroups in pre- and post-vaccination cfDNA integrity, Δ cfDNA integrity, or the percentages of cfDNA integrity-decreased cases were analyzed using the nonparametric Wilcoxon test.

according to the number of CTL response-positive peptides (a total of three groups including a zero-peptide group). The differences among the CTL response-positive and -negative groups or subgroups of the pre- and postvaccination cfDNA integrity or Δ cfDNA integrity were then analyzed. Again, no significant difference was found among the subgroups in each factor (Figure 6(E–G)).

The 130 patients or 58 patients, for whom we had complete data sets of IgG or CTL response, respectively, were divided into high and low cfDNA integrity groups at pre- and postvaccination, and the proportions of the two groups in two categories of vaccine-induced immune responses, i.e. response positive and negative, were analyzed using Fisher's exact probability test (Table 1). The 58 patients for whom we had complete data sets of both IgG and CTL responses were also divided into high and low cfDNA integrity groups at pre- and postvaccination, and the proportions of the two groups in four categories of vaccine-induced immune responses, i.e. $CTL^{+}IgG^{+}$, $CTL^{+}IgG^{-}$, $CTL^{-}IgG^{+}$, and $CTL^{-}IgG^{-}$, were analyzed using Fisher's test (Table 1). However, no significant correlation was observed between cfDNA integrity and vaccine-induced immune responses in any categories in either the pre- or postvaccination values.

**Table 1.** Relationship between cfDNA integrity and vaccine-induced immune responses.

| | | | Pre-cfDNA integrity | | Post-cfDNA integrity | |
|---|---|---|---|---|---|---|
| Vaccine-induced responses | | n | High | Low | High | Low |
| IgG response | | Total 130 | | | | |
| + | | 83 | 44 | 39 | 38 | 45 |
| − | | 47 | 21 | 26 | 27 | 20 |
| | Fisher's test | | $p = .46$ | | $p = .27$ | |
| CTL response | | Total 58 | | | | |
| + | | 26 | 15 | 11 | 12 | 14 |
| − | | 32 | 20 | 12 | 21 | 11 |
| | Fisher's test | | $p = .79$ | | $p = .18$ | |
| CTL and IgG response | | | | | | |
| CTL | IgG | Total 58 | | | | |
| + | + | 19 | 9 | 10 | 8 | 11 |
| + | − | 7 | 4 | 3 | 3 | 4 |
| − | + | 17 | 8 | 9 | 9 | 8 |
| − | − | 15 | 8 | 7 | 9 | 6 |
| | Fisher's test | | $p = 1.00$ | | $p = .77$ | |

## Discussion

Plasma cfDNA includes DNA derived from both the physiological death of normal cells and the pathological death of tumor cells [13]. Physiological cell death consists mostly of apoptosis, which causes DNA fragmentation; cfDNA fragments of such cells are generally <200 bp in length [14]. In contrast, pathologic cell death consists mainly of necrosis, and the cfDNA fragments of such cells are more random in size and include longer lengths [14]. The Alu element, which is the most abundant repetitive element in the human genome, is frequently used as a target sequence of cfDNA

integrity [12,15]. It is noted that there is no relationship between Alu and the vaccine peptides used in this study since Alu does not encode any proteins. The sequences of the vaccine peptides were derived from non-mutated proteins preferentially expressed in tumor cells [6,7].

The relationship between cfDNA integrity and disease progression or clinical stage of a tumor has been reported for various cancers including NSCLC [16–18]. We found that patients in the pre-vaccination cfDNA integrity high group survived longer than those in the low group (MST: 17.9 vs 9.0 months, HR: 0.58, $p = .0049$). A similar tendency was observed in post-vaccination cfDNA integrity (MST: 16.4 vs 9.4 months, HR: 0.65, $p = .024$). These results suggest that cfDNA integrity may be a prognostic biomarker in patients treated with the personalized peptide vaccine. An opposite result has been reported by another group; i.e. low pre-vaccination cfDNA integrity correlated with a favorable prognosis in patients with metastatic colorectal cancer treated with a combination of a cancer vaccine and chemotherapy as the first-line therapy [19]. Most of the difference between these two studies was the previous treatments. Our study population had a history of previous chemotherapy, whereas the other group's study population was chemotherapy-naïve. The cfDNA integrity of the naïve cases reflects only tumor size while that of the non-naïve cases reflects both tumor size and the effects of the latest chemotherapy and other therapies. The cfDNA integrity high group in our study might include the partial responders to the latest therapies. These different treatment backgrounds might explain the different results.

Plasma levels of cfDNA integrity were decreased after one vaccination cycle. Similar results were obtained in patients with advanced or recurrent ovarian cancer treated with the personalized peptide vaccine [20]. In that study, decreased levels of cfDNA integrity were correlated with vaccine-induced immune responses. However, no such correlation was observed for the NSCLC cases in the present study. It is unclear why the two studies showed different results, but a possible explanation is as follows: Recent studies regarding ICBs indicated the preexistence of antitumor immunity in tumor-bearing hosts and a majority of preexisting antitumor immunities recognizes neoantigens generated by tumor mutation [21]. Thus, the tumor mutation burden (TMB) might correlate with preexisting antitumor immunity. The TMB of NSCLC is ~10-fold that of ovarian cancer [22]. In fact, clinical studies of ICBs indicated NSCLC rather than ovarian cancer was the preferred target [23,24]. These findings suggest that preexisting immunity levels are higher in patients with NSCLC than in patients with ovarian cancer. Anti-tumor immunity levels in patients treated with vaccine therapy are a mixture of basal preexisting immunity and vaccine-induced immunity. Therefore, the influence of vaccination on cfDNA integrity may be lower in NSCLC patients than in ovarian cancer patients.

We have reported several biomarkers related to the prognosis of patients with various cancers treated with the personalized peptide vaccine [6–10]. The biomarkers were categorized as those related to (1) immune response, (2) inflammation, and (3) tumor cell death. Vaccine-induced CTL responses and IgG responses belong to the first category. Although the CTL response is a direct effect of the vaccination, it takes a long time to measure, and the measurement is neither easy nor robust. Therefore, we used IgG response as a surrogate immune marker [6–8]. Although the vaccine consisted of 9- or 10-mer CTL epitope peptides, some of the peptides also induced IgG responses when helper T-cells already existed in the patients. The early induction of the IgG responses was correlated with a better prognosis [6–11]. In the second category, plasma levels of C-reactive protein (CRP), interleukin (IL)-6, serum amyloid A (SAA), and C-C chemokine ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP-1) correlated with a poor prognosis [9, 25–29]. High-mobility group box 1 (HMGB1) is a damage-associated molecular pattern (DAMP) belonging to the third category, and the plasma levels of HMGB1 also correlated with a poor prognosis [30]. Circulating cfDNA contains both DNA derived from the physiological death of normal cells and the pathological death of tumor cells related to both tumor progression and anti-cancer treatments. Therefore, cfDNA integrity belongs to the third category.

Clinical studies of ICBs in patients with various cancers suggested the TMB correlated with ICB efficacy [31]. Nonsynonymous mutation of genes in tumor cells generates tumor-specific alteration of the amino acid sequences of the proteins, and some of those sequences will be recognized by CTLs as neoantigens in an HLA-restricted manner. Therefore, neoantigen vaccines are thought to be more specific and more effective than most of the classical peptide vaccines, including our personalized peptide vaccine, because they are targeted to nonmutated self-antigens preferentially expressed in tumor cell. Effector cells of both the classical and neoantigen vaccines are CTLs. Thus, our findings shown here are not specific to the classical peptide vaccines but might be applicable to neoantigen vaccines.

In conclusion, we investigated the plasma cfDNA integrity of patients with advanced NSCLC during treatment with a personalized peptide vaccination. We found that (1) the cfDNA integrity was decreased after the first cycle of vaccination and (2) the patients with high pre-vaccination cfDNA integrity survived longer than those with low pre-vaccination integrity (median survival time (MST): 17.9 vs 9.0 months, respectively; hazard ratio (HR): 0.58, $p = .0049$). A similar tendency was observed in post-vaccination cfDNA integrity (MST: 16.4 vs 9.4 months; HR: 0.65, $p = .024$). These results suggest that cfDNA integrity is a possible prognostic biomarker for patients with advanced NSCLC treated with cancer vaccines.

## Acknowledgments

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## Author contributions

KW and AY contributed to the study conception and design. Clinical sample collection was performed by KK and NT. Material preparation, data collection, and analysis were performed by KW, KM, and NK. The first draft of the manuscript was written by KW and AY, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Disclosure statement

Akira Yamada is a board member of the Bright Path Biotherapeutics (Kawasaki, Japan). The other authors have no conflict of interest.

## Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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