

# Prediction of the efficacy of immunotherapy by measuring the integrity of cell-free DNA in plasma in colorectal cancer

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We previously reported a phase II study of a cancer vaccine using five novel peptides recognized by *HLA-A\*2402*-restricted CTL in combination with oxaliplatin-containing chemotherapy (FXV study) as first-line therapy for patients with metastatic colorectal cancer and demonstrated the safety and promising potential of our five-peptide cocktail. The objective of this analysis was to identify predictive biomarkers for identifying patients who are likely to receive a clinical benefit from immunochemotherapy. Circulating cell-free DNA (cfDNA) in plasma has been reported to be a candidate molecular biomarker for the efficacy of anticancer therapy. Unlike uniformly truncated small-sized DNA released from apoptotic normal cells, DNA released from necrotic cancer cells varies in size. The integrity of plasma cfDNA (i.e. the ratio of longer fragments [400 bp] to shorter fragments [100 bp] of cfDNA), may be clinically useful for detecting colorectal cancer progression. We assessed plasma samples collected from 93 patients prior to receiving immunochemotherapy. The cfDNA levels and integrity were analyzed by semi-quantitative real-time PCR. Progression-free survival was significantly better in patients with a low plasma cfDNA integrity value than in those with a high value ( $P = 0.0027$ ). Surprisingly, in the *HLA-A\*2402*-matched group, patients with a low plasma cfDNA integrity value had significantly better progression-free survival than those with a high value ( $P = 0.0015$ ). This difference was not observed in the *HLA-A\*2402*-unmatched group. In conclusion, the integrity of plasma cfDNA may provide important clinical information and may be a useful predictive biomarker of the outcome of immunotherapy in metastatic colorectal cancer.

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths in industrialized countries.<sup>(1)</sup> Despite the availability of several effective cytotoxic drugs and new biological agents, the prognosis of metastatic colorectal cancer (mCRC) remains poor. The majority of patients are offered several lines of palliative chemotherapy, but these can expose patients to further side effects; as such, careful selection of patients and monitoring during therapy are essential.<sup>(2–6)</sup>

In an attempt to validate a new treatment modality to overcome the limited disease control of mCRC, we conducted a phase I vaccine trial<sup>(7)</sup> that confirmed the safety of a cocktail containing three peptides derived from oncoantigens (i.e. ring finger protein 43 [RNF43],<sup>(8)</sup> 34-kDa translocase of the outer mitochondrial membrane [TOMM34]<sup>(9)</sup> and IGF-II mRNA binding protein 3 [KOC1, also known as IMP-3],<sup>(10)</sup> and two peptides targeting vascular endothelial growth factor receptor 1 (VEGFR1)<sup>(11)</sup> and VEGFR2<sup>(12)</sup> for mCRC. We further conducted a phase II trial of a combination therapy with standard chemotherapy and five novel therapeutic peptides.<sup>(13)</sup> However,

because patients with very advanced-stage disease and who have a very poor immune status are also usually allowed to enroll into clinical studies during the early phase of drug development, it is very difficult to evaluate the survival benefit of treatment.<sup>(14)</sup> Hence, there is a desperate need for predictive biomarkers that allow the selection of suitable patients who are likely to respond well to the treatment. Better selection criteria in terms of predictive and prognostic markers to optimize treatment are needed.<sup>(15)</sup>

The level of circulating cell-free DNA (cfDNA) and the integrity of the cfDNA, which is defined as the ratio of long-base pair (bp) cfDNA/short-bp cfDNA, have been shown to be promising diagnostic biomarkers of colorectal and breast cancers.<sup>(16–19)</sup> Circulating cfDNA in the blood has attracted a great deal of attention as an easy-to-use tool for the evaluation of the malignant potential of various cancers.<sup>(20–22)</sup> Although circulating cfDNA is also present in healthy individuals, it has been implicated as a strong diagnostic and prognostic marker of malignancy. Its limitations are related to the contradictory findings reported regarding the proportion of tumor-derived

and non-tumor-derived cfDNA. Many different hypotheses concerning the origin of the circulating cfDNA have been considered, including active liberation by the tumor itself, the events of necrosis, apoptosis, mitotic catastrophe, autophagia, rupture of tumor cells, or circulation of micrometastases.<sup>(23)</sup> Among the various concepts of DNA liberation, most studies seem to be in accord with the notion that it occurs as a result of apoptosis or necrosis.<sup>(24)</sup> These two phenomena may be distinguished by the dimensions of the DNA fragments: the apoptotic death of cells causes the release of DNA fragments shorter than 200 bp into the circulation, whereas tumor necrosis is characterized by the presence of fragments that vary in size and are generally > 200 bp.<sup>(25)</sup> Hence, high cfDNA integrity may indicate a high degree of tumor cell collapse. In contrast, it might be better to show the data of circulating tumor DNA which express the tumor-related mutation. There are some major mutations in colorectal cancer, for example, *APC*, *TP53*, *KRAS* and *BRAF*. However, there is no common genetic mutation that is detected in all colorectal cancer.<sup>(26)</sup> Hence, in the current study, we investigated cfDNA integrity to determine the proportion of cfDNA from the tumors.

The aim of this study was to investigate whether cfDNA integrity may be a biomarker for response to immunochemotherapy in patients with mCRC.

## Materials and Methods

**Study design.** The detailed protocol of this study was described previously.<sup>(13)</sup> Briefly, we conducted a phase II trial that was a non-randomized, HLA-A status double-blinded study using five peptides recognized by *HLA-A\*2402*-restricted CTL: RNF43, TOMM34, KOC1 (IMP-3), VEGFR1 and VEGFR2. The therapy consisted of a cocktail of five therapeutic epitope peptides in addition to oxaliplatin-containing chemotherapy. Although the peptides used in this study were peptides recognized by *HLA-A\*2402*-restricted CTL, all enrolled patients, whose *HLA-A\*2402* status was double-blinded, were given the same regimen of peptide cocktail and oxaliplatin-containing chemotherapy. Between January 2009 and November 2012, 96 patients received the peptide cocktail treatment in combination with chemotherapy for advanced CRC or mCRC. Plasma samples were collected from 93 of the 96 patients prior to treatment. This study was carried out in accordance with the Helsinki Declaration on experimentation involving human subjects, was approved by the institutional ethics review boards of Yamaguchi University (H20-102) and each study site, and was registered in the UMIN Clinical Trials Registry (UMIN000001791).

**Patients and plasma.** Pretreatment plasma samples were available for cfDNA analysis from 93 patients (*HLA-A2402*-matched,  $n = 49$ ; and *HLA*-unmatched,  $n = 44$ ) with mCRC who were treated in the phase II study. Peripheral blood from each patient was collected in EDTA tubes. The blood samples were centrifuged at 400 *g* for 15 min at 4°C. The plasma was then aliquoted and stored at -80°C until use.

**DNA strand integrity analysis.** DNA was purified from 500  $\mu$ L of plasma with a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) was used to measure the DNA concentrations according to the manufacturer's instructions. DNA strand integrity was measured using semi-quantitative real-time PCR with LightCycler (Roche, Penzberg, Germany) to determine the integrity index, which was defined as the ratio of the relative abundances of 400-bp versus 100-bp

PCR products of the  $\beta$ -actin gene that are likely present in all normal and neoplastic cells. Both 100- and 400-bp PCR fragments were amplified using the same forward primer: 5'-GCACCACACCTTCTACAATGA-3'. The nested reverse primers used were 5'-GTCATCTTCTCGCGTTGGC-3' and 5'-TGTCACGCACGATTCCC-3' for the 100-bp and 400-bp products, respectively. The PCR conditions were: denaturation for 3 min at 95°C followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 57°C (100 bp) or 56°C (400 bp) for 30 s, and extension at 72°C for 10 s (100 bp) or 15 s (400 bp). The threshold ( $C_T$ ) value for each reaction was calculated by the iCycler software package. The  $\Delta C_T$  value for 400 bp was subtracted from that for 100 bp to obtain a  $\Delta\Delta C_T$  value. The integrity index was calculated as the exponential of  $(-\Delta\Delta C_T \times \text{LN } 2)$ .

**Statistical analysis.** Overall survival (OS) and progression-free survival (PFS) rates were analyzed using the Kaplan–Meier method, and survival was measured in days from the first vaccination to the day of patient death from any cause. *P*-values were assessed using a log-rank test. A Cox's proportional hazards model and a logistic regression model were used to estimate the hazard ratios (HR) for the treatment effect in relation to survival and biomarkers or prognostic clinical information. All statistical analyses were performed with SPSS Statistics 20.0 (SPSS, Chicago, IL, USA). A value of  $P < 0.05$  was considered to be statistically significant.

## Results

**Patient characteristics.** The clinical characteristics of the patients are shown in Table 1. None of the baseline characteristics differed significantly between the *HLA*-matched and *HLA*-unmatched groups.

**Table 1. Patient characteristics**

Characteristic	HLA-A*2402		P-value
	Matched ( $n = 49$ )	Unmatched ( $n = 44$ )	
Age	64.1 $\pm$ 1.5	63.4 $\pm$ 1.2	NS
Sex			
Male	25	22	NS
Female	24	22	
Unresectable metastatic organ			
Liver	27	34	NS
Lung	18	12	
Peritoneum	5	4	
Lymph node	12	12	
Others	5	1	
Number of involved organs			
1	35	29	NS
2	9	10	
3	5	5	
Resection of primary lesion			
Yes	40	41	NS
No	9	3	
Tumor marker			
CEA	1220 $\pm$ 684	341 $\pm$ 86	NS
CA19-9	3609 $\pm$ 2157	4506 $\pm$ 3457	

Data are expressed as mean  $\pm$  standard error. CA19-9, carbohydrate antigen; CEA, carcinoembryonic antigen; HLA, human leukocyte antigen; NS, not significant.

**Table 2. Results of cell-free DNA levels before treatment**

Marker	HLA-A*2402	
	Matched (n = 49)	Unmatched (n = 44)
Cell-free DNA amount (ng/mL plasma)	25.10 ± 3.59	22.78 ± 3.49
Integrity (400-bp/100-bp ratio)	0.37 ± 0.03	0.28 ± 0.02

Data are expressed as mean ± standard error. HLA, human leukocyte antigen.

**cfDNA levels and cfDNA integrity.** The pretreatment levels of cfDNA and the calculated cfDNA integrity of the 93 patients are shown in Table 2.

**cfDNA levels and objective responses.** In this study, we categorized the patients into two groups: those with a cfDNA level higher than the median and those with a value lower than the median. The objective response rate (ORR) was 63.2 and 61.3% in the *HLA*-matched and *HLA*-unmatched groups, respectively ( $P = 0.910$ ). The proportion of patients with a complete response, partial response and stable disease was 2.0% (1/49), 61.2% (30/49) and 32.6% (16/49), respectively, in the *HLA*-matched group, and 0% (0/44), 61.3% (27/44) and 38.6% (17/44), respectively, in the *HLA*-unmatched group. There was no significant difference in ORR between patients with a high or low cfDNA level. Similarly, there was no

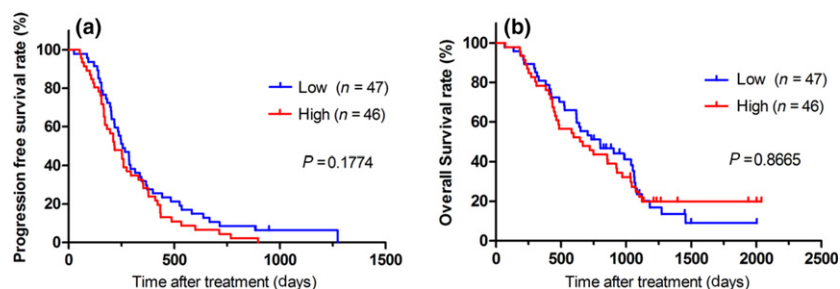
significant difference in the integrity of cfDNA between patients with a high or low cfDNA level (data not shown).

**cfDNA levels and survival.** In this study, we categorized the patients into two groups according to the amount of cfDNA, as described above. There was no significant difference in PFS (Fig. 1a) or OS (Fig. 1b) between patients with a high or low cfDNA level.

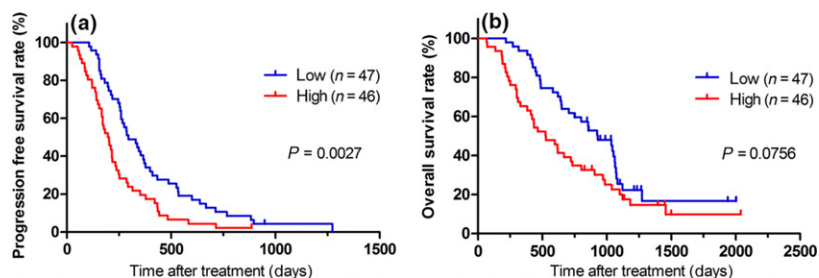
**cfDNA integrity and survival.** Next, we also categorized the patients into two groups according to the integrity of cfDNA: those with a cfDNA integrity value higher than the median and those with a value lower than the median. We found that a low cfDNA integrity value was a prognostic marker for a longer PFS with peptide cocktail and oxaliplatin-containing chemotherapy (Fig. 2a,  $P = 0.027$ ). In contrast, there was no significant difference in OS between those with a high or low cfDNA integrity value, although the OS tended to be slightly better in the group with a low cfDNA integrity value ( $P = 0.0756$ , Fig. 2b).

Surprisingly, in the *HLA-A\*2402*-matched group, patients with a low plasma cfDNA integrity value had a significantly better PFS than those with a high value ( $P = 0.0015$ ; Fig. 3a). In contrast, in the *HLA-A\*2402*-unmatched group, there was no difference between the patients with a high or low cfDNA integrity value (Fig. 3b). Hence, cfDNA integrity might be a predictive biomarker of PFS in patients treated with peptide vaccines. Multivariate analysis of the Cox regression model

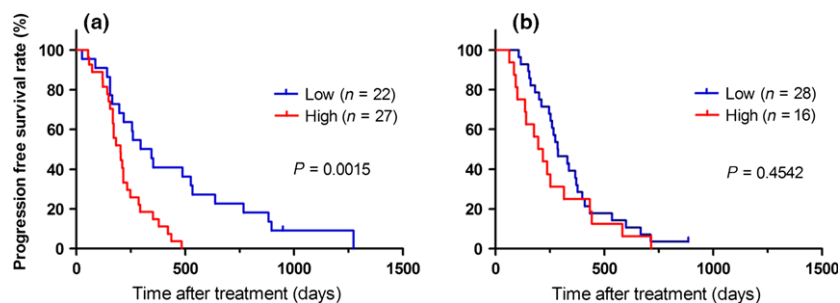
**Fig. 1.** Kaplan–Meier survival plots of progression-free survival (PFS) and overall survival (OS) in patients with a plasma cell-free DNA level higher or lower than the median. (a) PFS. (b) OS.



**Fig. 2.** Kaplan–Meier survival plots of progression-free survival (PFS) and overall survival (OS) in patients with a plasma cell-free DNA integrity value higher or lower than the median. (a) PFS. (b) OS. The log-rank test indicated that cell-free DNA integrity might be a predictive biomarker for PFS ( $P = 0.0027$ ).



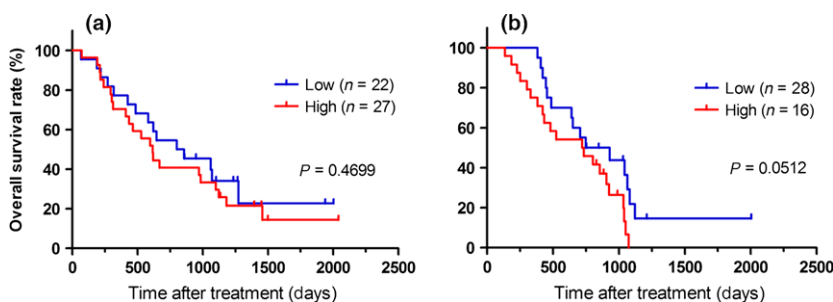
**Fig. 3.** Kaplan–Meier survival plots of progression-free survival (PFS) in patients with a plasma cell-free DNA integrity value higher or lower than the median. (a) *HLA-A\*2402*-matched group. (b) *HLA-A\*2402*-unmatched group. In the *HLA-A\*2402*-matched group, cell-free DNA integrity might be a predictive biomarker for PFS ( $P = 0.0015$ ).



**Table 3. Univariate and multivariate analysis of biomarkers for progression free survival using Cox regression model**

Factor	Cut-off	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
CEA (ng/mL)	$\geq 2 \times \text{ULN}$	1.21	0.63–2.23	0.5433			
CA19-9 (U/mL)	$\geq 2 \times \text{ULN}$	0.95	0.51–1.85	0.8885			
CRP (mg/dL)	$\geq 1$	1.2	0.60–2.28	0.5799			
Number of involved organs	$\geq 2$	1.32	0.66–2.46	0.4064			
cfDNA	$\geq \text{median}$	1.24	0.69–2.22	0.4644			
cfDNA integrity	$\geq \text{median}$	2.73	1.42–5.48	0.0023	2.64	1.34–5.44	0.0045

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CI, confidence interval; cfDNA, cell free DNA; CRP, C-reactive protein; HR, hazard ratio; ULN, upper normal of limit.



**Fig. 4.** Kaplan–Meier survival plots of overall survival (OS) in patients with a plasma cell-free DNA integrity value higher or lower than the median. (a) *HLA-A\*2402*-matched group. (b) *HLA-A\*2402*-unmatched group.

indicated that cfDNA integrity was the most significant predictor for PFS in the *HLA-A\*2402*-matched group [ $P = 0.0037$ ; HR = 2.66; 95% confidence interval (CI) = 1.34–5.44, Table 3]. In contrast, in both the *HLA-A\*2402*-matched (Fig. 4a) and *HLA-A\*2402*-unmatched (Fig. 4b) groups, there was no significant difference in OS between the patients with a high or low cfDNA integrity value.

## Discussion

Preservation of the host immune system is essential for active specific immunotherapy. Hence, it is crucially important to explore biomarkers for predicting the clinical responses for successful immunotherapy.<sup>(27)</sup> We have investigated possible predictive biomarkers for the efficacy of immunochemotherapy based on clinical information and patient samples from our previous phase II study of a therapeutic peptide cocktail vaccine in combination with an oxaliplatin-containing chemotherapy regimen against mCRC.<sup>(7,13,15)</sup>

To the best of our knowledge, this is the first study to report that cfDNA integrity might be a predictive biomarker for the efficacy of immunochemotherapy (Figs 2 and 3). In the present study, the PFS was significantly better in patients with a low cfDNA integrity value than in patients with a high value ( $P = 0.0027$ , Fig. 2a); surprisingly, this significance was observed clearly only in the *HLA-A\*2402*-matched group ( $P = 0.0015$ , Fig. 3a), and not in the *HLA-A\*2402*-unmatched group ( $P = 0.4542$ , Fig. 3b). These results indicated that the cfDNA integrity value is a predictive biomarker for peptide vaccines, but not for chemotherapy alone.

The immunogenic strength of apoptotic or necrotic tumor cells has been reported, controversially, to induce antitumor immunity.<sup>(28,29)</sup> In the tumor microenvironment, necrosis or necroptosis<sup>(30)</sup> has been reported to result in the release of damage-associated molecular pattern molecules, causing strong immunosuppression through the induction of regulatory T cells,

myeloid-derived suppressor cells and mesenchymal stromal cells.<sup>(31)</sup> The plasma cfDNA integrity value may be determined from a liquid biopsy to remotely detect the amount of necrosis/necroptosis in tumors and the immunosuppressive microenvironment of the tumor site for predicting the efficacy of immunotherapy with peptide vaccines. A high cfDNA integrity value may indicate a high degree of tumor collapse and an excess of tumor necrosomes, which promote inducible immune suppression, leading to resistance to immunotherapy and a shorter PFS.<sup>(32)</sup> Moreover, the half-life period of cfDNA was reported as several minutes to several hours in the peripheral blood.<sup>(33–35)</sup> Hence, the cfDNA integrity may reflect the process occurring within a living organism in real-time.

In contrast, there was no significant difference in OS between the patients with a high or low cfDNA integrity value (Fig. 2b). In general, OS is a preferable endpoint to PFS due to the delayed effect of vaccine therapy.<sup>(13)</sup> It was speculated that the tumor microenvironment might be altered after chemotherapy due to the large amount of necrosis in the tumor, which results in the release of damage-associated molecular pattern molecules.<sup>(31,32)</sup> Most of the current treatment strategies for cancer (chemotherapy, radiation therapy and hormonal therapy) promote the release of damage-associated molecular pattern molecules following therapy-induced tumor death by necroptosis and necrosis. Regulatory T cells are strongly affected by pattern recognition receptor signaling in the tumor microenvironment and they limit immune reactivity in coordination with myeloid-derived suppressor cells. To overcome the limitations of combination immunochemotherapy, some additional therapy may be needed to regulate the immunosuppressive tumor microenvironment. Several commonly used drugs, such as cyclophosphamide,<sup>(36,37)</sup> COX-2 inhibitor,<sup>(38)</sup> metformin<sup>(39)</sup> and cimetidine,<sup>(40)</sup> have been shown to modify the suppressive immune status in tumor microenvironments and might enhance the immune responses induced by peptide vaccines.



In conclusion, the plasma cfDNA integrity value might be a useful predictive biomarker for the outcome of immunotherapy, and an immunomodulating agent should be included in combination immunochemotherapy.

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