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

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Key words
Biomarker, cell-free DNA, colorectal cancer, DNA integrity, immunotherapy

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Funding Information
Shoichi Hazama received research funding from NEC Corporation and Toyo Kohan Corporation. Shigefumi Yoshino received honoraria from MSD Corporation for lectures.

Received July 15, 2016; Revised September 18, 2016; Accepted September 21, 2016

Cancer Sci 107 (2016) 1825–1829
doi: 10.1111/cas.13085

C oral cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths in industrialized countries. 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.

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

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. 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. 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, autophagic rupture of tumor cells, or circulation of micrometastases. 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. 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. 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 cancers. 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. 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-A^2402-matched, n = 49; and HLA-A^2402-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 μL 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 β-actine 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’-GCACCACACCTTCTACATGA-3’. The nested reverse primers used were 5’-GTCTACTTCTGCGGTGTCG-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 ΔC_T value for 400 bp was subtracted from that for 100 bp to obtain a ΔΔC_T value. The integrity index was calculated as the exponential of (−ΔΔC_T × 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 ± 1.5          | 63.4 ± 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                   | 5       | 1        |
| Number of involved organs      |                     |         |         |
| 1                              | 8                   | 12      | NS       |
| 2                              | 9                   | 10      |         |
| 3                              | 5                   | 5       |         |
| Resection of primary lesion    |                     |         |         |
| Yes                            | 40                  | 41      | NS       |
| No                             | 9                   | 3       |         |
| Tumor marker                   |                     |         |         |
| CA19-9                         | 1220 ± 684          | 341 ± 86 | NS       |
| Data are expressed as mean ± standard error. CA19-9, carbohydrate antigen; CEA, carcinoembryonic antigen; HLA, human leukocyte antigen; NS, not significant. | 3609 ± 2157 | 4506 ± 3457 |
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.

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...
necroptosis(30) 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.(31) 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.(32) Moreover, the half-life period of cfDNA was reported as several minutes to several hours in the peripheral blood.(33,35) 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.

**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.(27) 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.(7,13,15)

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.(28,29) In the tumor microenvironment, necrosis or necroptosis(30) has been reported to result in the release of damage-associated molecular pattern molecules, causing strong immunosuppression through the induction of regulatory T cells, HLA-A*2402 = HR cfDNA integrity
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.

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

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

Acknowledgments

This study was supported partially as part of a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct), The Japan Agency for Medical Research and Development (AMED).

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Disclosure Statement

Shoichi Hazama received research funding from NEC Corporation and Toyo Kohan Corporation. Shigefumi Yoshino received honoraria from MSD Corporation for lectures. The other authors have no conflict of interest to declare. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

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