Abstract. Background: We previously reported a phase I study of a cancer vaccine using five novel HLA-A*2402-restricted peptides, and demonstrated the safety and the promising potential of our five-peptide cocktail for advanced colorectal cancer. The objective of this analysis was to investigate predictive biomarkers for the prior selection of patients who are likely to have clinical benefit from such therapy. Patients and Methods: Seventeen patients with colorectal cancer who were treated with the five peptides underwent a complete blood count, serum chemistry tests and enzyme-linked ImmunoSpot assay before the treatment as predictive markers of high reactivity to the peptides. Results: Interleukin-6 level was a significant predictor for overall survival of patients treated with the peptide cocktail \( p=0.017 \). A high neutrophil/lymphocyte ratio was likely to have some association with the poor induction of peptide-specific immune reaction. Conclusion: Interleukin-6 level might be a good predictive biomarker for clinical benefit of patients treated with this peptide vaccine.

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Tumor-associated antigens (TAAs) were reported in the early 1990s (1). Since then, various approaches to discovering TAAs have been reported (2). Among them, genome-based technologies, including comprehensive gene expression profiles of malignant cells, have contributed very significantly to the identification of cancer-specific antigens (3-7). We previously reported the identification of three oncoantigens that showed cancer-specific expression patterns and have oncogenic activities, ring finger protein 43 (RNF43) (4), 34 kDa translocase of the outer mitochondrial membrane (TOMM34) (5), and insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3, other name is KOC1) (6), as targets for the development of cancer peptide vaccines for colorectal cancer (CRC).

Active immunotherapy using these epitope peptides, aiming at in vivo induction of tumor-specific cytotoxic T-lymphocytes (CTLs), requires the preservation of the host immune system, as recommended in the guidance for therapeutic cancer vaccines released from the US Food and Drug Administration in 2011 (8). However, since patients with very advanced stage disease whose immune status is very poor are usually allowed to enroll to clinical studies in an early phase of drug development, it is very difficult to evaluate their survival benefit (9). Hence, there is a desperate need for predictive biomarkers to allow the prior selection of patients who are likely to respond well and in whom CTLs would be induced effectively by epitope peptides.

We previously reported a phase I study of a combination vaccine treatment using a cocktail containing three peptides derived from oncoantigens and two peptides targeting
vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 (10) for metastatic CRC, and reported its safety and its promising potential for inducing CTLs, and prolonging overall survival (10). Although peptide-specific CTL induction and skin reactions at the vaccine injection site might be good markers for monitoring immune responses, a few months are required for skin reactions to become apparent, and CTL measurement is also time-consuming and not very quantitative. The purpose of this study was to explore predictive biomarkers for the efficacy of immunotherapies before treatment and for the selection of patients likely to exhibit better treatment outcomes following vaccination. We herein demonstrate possible predictive biomarkers for active immuntherapies.

**Patients and Methods**

*Patients and study design.* The detailed protocol of this study was described previously (10). Briefly, patients were eligible for enrollment when they had histologically confirmed CRC without indication of surgical resection, when they had failed to respond to prior standard chemotherapy or were intolerable to the standard therapy, and when they were HLA-A*2402-positive by DNA typing. The patients were required to have an Eastern Cooperative Oncology Group performance status (PS) of 0 to 2, a life expectancy of at least 3 months, and to have adequate organ functions. This study was approved by the Institutional Ethics Review Boards of Yamaguchi University (H18-82) and was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent prior to study entry. The study protocol was registered at the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN000004948).

This study was primarily conducted to evaluate the safety and to find the recommended dose of these peptides. Good Manufacturing Practice grade peptides of RNF43-721 (NSQPVWLCL) (11), TOMM34-299 (KLRQEVKQNL) (5), KOC1 (IMP-3)-508 (KTVPNELQNL) (12), VEGFR1-1084 (SYGVLLWEI) (13) and VEGFR2-169 (RFVPDGNRI) (14) peptides restricted with HLA-A*2402 were synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA). Dose escalation was performed in three patient cohorts with doses of 0.5, 1, and 3 mg for each peptide. Each peptide was mixed with 0.5 ml of incomplete Freund’s adjuvant (IFA) (Montanide ISA51; Seppic, Paris, France) administered to patients subcutaneously into the thigh or axilla regions on days 1, 8, 15, and 22 in a 28-day treatment course. According to the result of this study, we decided the recommended dose of each peptide for further study to be 3.0 mg, adding three patients at the dose of 3.0 mg to confirm the safety. Next we performed a single injection of the cocktail of five peptides which could be expected to induce immune responses at the same level as separate injections of each of the five peptides. The cocktail of five peptides at the dose of 3 mg was mixed with 1.5 ml of IFA and administered to six patients. Vaccination was continued even after the progression of disease when a patient wished and a primary doctor who provided best supportive care or additional chemotherapies recommended. From the fourth course of treatment, the vaccination schedule was changed to be biweekly, and from the seventh course, it was reduced to once a month.

The study confirmed the safety as well as immunological and antitumor effects in 18 patients. A patient who underwent curative resection after eight weeks of vaccination was excluded from the analysis; hence seventeen patients were included in the analysis of the present study.

**Sample collection.** A complete blood count and serum chemistry tests were performed before treatment and every two weeks. Fifty milliliters of blood was drawn before each course, and then peripheral-blood mononuclear cells (PBMCs) and blood plasma were isolated. PBMCs and plasma were preserved in liquid nitrogen until examination.

**Measurement of the peptide-specific IFN-γ response and plasma IL6 level.** Antigen-specific T-cell response was estimated by enzyme-linked ImmunoSpot (ELISPOT: CTL, Shaker Heights, OH, USA) assays following *in vitro* sensitization as described previously (10, 15). The number of peptide-specific spots was calculated by subtracting the spot number in the control well from the spot number of a well with vaccinated peptide-pulsed stimulator cells. Antigen-specific T-cell response was classified into four grades (−, +, ++, or ++++) according to the algorithm flow chart described in our previous report (+++: IFN-γ producing cells more than 0.2%, ++: 0.02-0.2%, +: 0.01-0.02%, −: less than 0.01% in the sample applied for ELISPOT). Plasma IL6 level was measured by electrochemiluminescence immunoassays (Meso Scale Discovery, Rockville, MD, USA) according to the manufacturer’s instructions.

**Statistical analysis.** Overall survival (OS) rates were analyzed by 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 (HRs) for the treatment effect in relation to OS and biomarkers. Student’s *t*-test was used for the analysis of peptide-specific immune responses. All statistical analyses were performed with SPSS statistics 20.0 (SPSS, Chicago, IL, USA). A value of *p*<0.05 was considered statistically significant.

**Results**

As described previously (10), the vaccine treatment using multiple peptides was well-tolerated without any severe treatment-associated systemic adverse events. According to the clinical outcome, one patient achieved complete response and six patients had stable disease for four to seven months. The median overall survival time (MST) was 13.5 months. Patients in which we detected induction of CTLs specific to three or more peptides had a significantly better prognosis (MST=27.8 months) than those with poorer immune responses (MST=3.7 months) (*p*=0.032).

**Immunological parameters as biomarkers of immunotherapy.** To explore biomarkers for this vaccine therapy, we analyzed immunological parameters of the 17 patients. Univariate analysis revealed that 15% or more lymphocytes in white blood cells in peripheral blood (*p*=0.042), a neutrophil/lymphocyte ratio (NLR) less than 5 (*p*=0.042), C-reactive protein (CRP) less than 1.0 mg/dl (*p*=0.022), and plasma IL6
level of less than 2.0 pg/ml ($p=0.015$) might be biomarkers predictive of better prognosis of patients treated with the peptide cocktail (Table I). Multivariate analysis of the Cox regression model indicated that IL6 was the most significant predictor for OS ($p=0.017$; HR=4.212; 95% confidence interval (CI)=1.289 to 13.756, Table II). The log-rank test also indicated that IL6 of <2.0 pg/ml might be a predictive biomarker for OS ($p=0.009$; Figure 1).

Relationship to CTL induction. The relationships between CTL induction and lymphocyte proportion, NLR, and CRP or IL6 level were evaluated. A low lymphocyte proportion and a high NLR were related to lower induction of a peptide-specific immune reaction ($p=0.061$), although a high CRP level and a high IL6 concentration had no effect on CTL induction (Table III).

Discussion

The preservation of the host immune system is critically essential for active specific immunotherapy aiming in vivo induction of CTLs (16) and long-term vaccination is required to observe CTL induction and clinical benefit (8). Hence, it is crucially important to explore biomarkers for predicting the host’s immunity and the clinical responses for successful immunotherapy (17).

We investigated possible predictive biomarkers for immunotherapy using the information of our previous phase I study of a combination vaccine treatment for metastatic CRC with a cocktail consisting of three peptides derived from oncoantigens and two peptides targeting VEGFR1 and VEGFR2 (10), and found interesting correlations between certain parameters and clinical outcome.

Firstly, a plasma IL6 level of 2.0 pg/ml or more might be the most useful predictor for poor OS by multivariate analysis of the Cox regression model ($p=0.017$; HR=4.212; Table II), and the log-rank test also suggested that patients with an IL6 level of 2.0 pg/ml or more had shorter OS than those with a lower IL-6 ($p=0.009$), (Figure 1). However, induction of CTLs was not affected by the plasma IL6 levels (Table III), implying that IL6 might modulate the local immune response and protect cancer cells from immune attack by CTLs. The tumor stromal cells consist of a variety of cellular components, including various kinds of immune-associated and inflammatory cells, e.g. regulatory T-cells, tumor-associated macrophages, myeloid suppressor cells, which release chemokines and cytokines including IL6, thus inhibiting antitumor immunity and leading to tumor resistance (18, 19). These results indicate that the regulation of the chronic inflammation surrounding tumor tissues using selective blockade of IL6 trans-signaling (20) or monoclonal antibody against IL6 (21) may be a good approach for further improving the clinical efficacy of vaccination.
Secondly, an NLR of 5.0 or more and a lymphocyte proportion less than 15% \( (p=0.042 \text{ for both}) \), \( \text{Table I} \) appear to be predictive of poor OS according to the univariate analysis. Moreover, patients with an NLR of 5.0 or more and a lymphocyte proportion less than 15% \( (p=0.065 \text{ for both}) \), \( \text{Table III} \) exhibited poor induction of CTLs, suggesting that these patients are unlikely to have clinical benefit of active immunotherapy.

In conclusion, IL6 may be the most promising biomarker for predicting the prognosis of patients treated with peptide vaccines and a combination of anti-IL6 agents with cancer vaccination may improve the clinical benefit of immunotherapy. Furthermore, although the data are very preliminary, patients with a lymphocyte proportion less than 15% or NLR of more than 5.0 may not expect clinical benefit of active immunotherapy.

**Disclosure**

Yusuke Nakamura is a stock holder and a scientific advisor of OncoTherapy Science, Inc. The other Authors have no potential conflicts of interest to disclose.

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