Abstract. Cancer vaccines have been developed as a new therapeutic approach, however, their clinical benefit remains limited. We previously performed a phase II study for advanced colorectal cancer (CRC) using five human leukocyte antigen (HLA-A*24:02)-restricted peptides derived from kinase of the outer chloroplast membrane 1, translocase of outer mitochondrial membrane 34 (TOMM34), ring finger protein 43 (RNF43), vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2. In the present study the relationship between overall survival (OS) and several biomarkers, including cytotoxic T lymphocyte (CTL) and immunoglobulin G (IgG) responses to these five peptides, was investigated. In 89 advanced CRC patients treated with a combination therapy consisting of these five peptides and oxaliplatin-based chemotherapy, plasma was collected before and after 3 months of vaccine administration. IgGs reactive to each of the five peptides were assessed using the multiplex bead suspension Luminex system. Antigen-specific T-cell responses were estimated by enzyme-linked immunoSpot assay. Plasma levels of TOMM34 IgG (P<0.001), RNF43 IgG (P<0.001) and VEGFR2 IgG (P<0.001) were significantly increased after vaccination and stronger VEGFR2 IgG responses correlated significantly with OS in HLA-matched patients (P=0.034). CTL responses to VEGFR1 and VEGFR2 were also significantly increased in the HLA-matched group (P=0.049 and P<0.001, respectively). However, increased CTL response did not correlate with OS. Multivariate analysis indicated that IgG responses to VEGFR2 were the most significant predictor for OS in the HLA-A*24:02-matched group (P=0.04). Our findings indicated that VEGFR2 IgG responses may be an important immunological biomarker in the early course of treatment for CRC patients treated with therapeutic epitope peptides.
cause of cancer-related deaths. In 2012, 134,575 new cases of CRC occurred, while 48,785 patients succumbed to this disease in 2014, accounting for 13.3% of all cancer-related deaths in Japan that year (2). In the past decade, the development of several effective cytotoxic drugs and molecular targeted drugs and their combinations, has notably improved the prognosis of patients with metastatic CRC (mCRC) (3-5). However, many patients present with disease progression due to chemotherapy resistance.

For the purpose of overcoming the limitations of mCRC treatment, we previously conducted a phase II study for the unresectable CRC using five human leukocyte antigen (HLA-A*24:02)-restricted peptides (6), three derived from oncoantigens [kinase of the outer chloroplast membrane 1, KOC1 (7); translocase of outer mitochondrial membrane 34, TOMM34 (8); and ring finger protein 43, RNF43 (9)] and two derived from vascular endothelial growth factor receptors [VEGFR1 (10) and VEGFR2 (11)]. In that study, we demonstrated a significant improvement in patient survival 20 months after the beginning of the vaccination. However, it is extremely difficult to evaluate the clinical benefit of treatment because patients with advanced cancer and with poor immunity are generally admitted to enroll into clinical trials during the initial stage of drug development (12). Therefore, it is necessary to identify predictive biomarkers that facilitate the selection of patients who are likely to respond well, either before treatment or early in the course of treatment (13). Although CEA, CA19-9 and CRP are included as predictive and prognostic biomarkers in CRC (14-16), they are not necessarily adapted to immunotherapy. To this purpose, we have previously reported candidate biomarkers of efficacy for such peptide-vaccine treatments (17-19).

In spite of the fact that both cellular and humoral immune responses are important for the induction of potent antitumor immunity, most peptide-vaccine studies have focused only to cellular responses (20). Under these circumstances, it was reported that immunoglobulin G (IgG) responses were identified as a remarkable biomarker for predicting the overall survival (OS) of vaccinated patients (20,21). In the present study, the relationship between OS and potential biomarkers of efficacy, including cytotoxic T lymphocyte (CTL) and IgG responses to the vaccinated peptides, were investigated in patients with advanced CRC.

Materials and methods

Patients and eligibility criteria. The detailed protocol of this phase II study was previously described (6). Briefly, patients were eligible for enrollment if they were histologically confirmed as advanced CRC, were chemotherapy-naive, had adequate functioning of critical organs and had a life expectancy of ≥3 months. Written informed consent was obtained from all patients prior to their entry into the present study. This study was approved by the Institutional Ethics Review Boards of Yamaguchi University (H20-102) and was registered in the UMIN Clinical Trials Registry as UMIN000001791.

This was a single-arm trial, non-randomized and HLA-A status double-blind study to evaluate the clinical benefits of cancer vaccination treatment for advanced CRC. The therapy consisted of a cocktail of five therapeutic HLA-A*24:02 restricted epitope-peptides: RNF43-721 (NSQPVWLCL) (9), TOMM34-299 (KLQEVKQNL) (8), KOC1 (IMP-3)-508 (KTVNELONL) (22), VEGFR1-1084 (SYGGVLLWEI) (23) and VEGFR2-169 (RFVPDGNR1) (11) in addition to oxaliplatin-containing chemotherapy.

The cocktail containing 3 mg of each of the five peptides was mixed with 1.5 ml of incomplete Freund's adjuvant (IFA, Montanide ISA51; Seppic, Paris, France) and administered subcutaneously into the thigh or axilla region on day 1 of each week for 13 weeks and then the vaccination schedule was reduced to once every 2 weeks.

Oxaliplatin-containing regimens were administered concurrently with the peptide vaccinations. Briefly, mFOLFOX6 (24) consisted of oxaliplatin (85 mg/m²) with leucovorin (400 mg/m²), followed by a FU (400 mg/m²) bolus, and then 2,400 mg/m² continuous infusion without bevacinumab. This treatment was repeated biweekly. XELOX (25) consisted of oxaliplatin (130 mg/m²) on day 1, followed by oral capecitabine (1,000 mg/m²) twice daily on day 1 through 14 of a 21-day cycle without bevazumab.

Sample collection. Complete blood counts and serum chemistry tests were performed before the treatment and every two weeks thereafter. A total of 15 ml of blood were drawn before each course and then peripheral-blood mononuclear cells (PBMCs) and blood plasma were isolated by means of Ficol-Conray density gradient centrifugation. The evaluation of PBMCs viability was performed by Vi-CEL™ XR (Beckman Coulter, Inc., Brea, CA, USA). PBMCs and plasma were preserved in liquid nitrogen until examination.

Assessment of peptide-specific CTL responses. Anti-gene-specific T-cell responses were assessed by enzyme-linked ImmunoSpot (ELISpot) assay following in vitro sensitization, as previously described (26). Briefly, frozen PBMCs derived from the patient were thawed concurrently and viability was confirmed as >90%. PBMCs (5x10⁵/ml) were cultured with 10 mg/ml of the candidate peptide and 100 IU/ml of interleukin (IL)-2 (Novartis, Emeryville, CA, USA) at 37°C for 2 weeks. Peptide was added into the culture on day 0 and 7. To monitor antigen-specific immune responses, ELISpot assays were performed with the human IFN-γ ELISpot PLUS kit (Mabtech AB, Nacka Strand, Sweden). Briefly, 96-well plates with nitrocellulose membranes (Millipore, Molsheim, France) were pre-coated with primary anti-IFN-γ antibody (1-DIK) at 4°C overnight. The plates were then pre-reacted with RPMI-1640 medium containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc, Waltham, MA, USA).

For the HLA-matched group, vaccine peptide (10 µg/ml)-pulsed or HIV-specific peptide (RYLRDQQLL, 10 µg/ml)-pulsed (as the control) HLA-A*24:02-positive TISI cells (IHGW Cell and Gene Bank, Seattle, WA, USA) (2x10⁶/well) were used as stimulators and incubated for 24 h with responder cells (from 2x10⁵/well to 2.5x10⁶/well in triplicate, in a total of 200 µl/well), at different responder/stimulator ratios as indicated. Stimulation with phorbol 12-myristate 13-acetate (PMA, 25 ng/ml) plus ionomycin (500 µM) (both from Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control for T-cell activity.
For the HLA-unmatched group, responder cells (2x10^4/well) and each peptide (10 µg/ml) or HIV-specific peptide (10 µg/ml), in a total of 200 µl/well, were cultured in triplicate without stimulators for 24 h again using the combination of PMA and ionomycin as a positive control.

For subsequent measurements, these cell mixtures were treated with biotinylated secondary anti-IFN-γ antibody (7-B6-1) and incubated for 2 h. Then the plates were incubated with HRP-reagent and stained with TMB (Mabtech AB). Protein spots were quantified with an auto-analysis system, ImmunoSPOT S4 (Cellular Technology Ltd., Cleveland, OH, USA). Antigen-specific T-cell responses were estimated as previously described (27), and classified into four grades (+, ++, +++ and ++++) according to an algorithm described in a previous study (28). Antigen-specific T-cell responses classified (+) or more, were defined as CTL positive.

Assessement of peptide-specific IgG responses. The levels of anti-peptide IgGs were assessed using the Luminex system (Austin, TX, USA), as previously reported (29). In brief, 100 µl of 1:100 diluted plasma were incubated with 25 µl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 µl of biotinylated goat anti-human IgG (γ-chain specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 µl streptavidin-phycocerythrin (PE)/well and incubated for an additional 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 µl of Tween-phosphate buffered saline (PBS) into each well. Each sample (50 µl) was then analyzed using the Luminex system. Samples with antigen-specific IgG values at least 4-fold higher than those prior to treatment, were defined as being IgG positive.

Peptide-binding assays. The binding of peptides to the HLA-A*24:02 molecule was determined by acid stripping and a reconstitution assay, as previously described by Zeh et al (30) with minor modifications. Briefly, C1R-A24 cells were exposed to pH 3.3 citrate phosphate buffer and were then reconstituted with minor modifications. C1R-A24 cells were treated with biotinylated secondary anti-IFN-γ antibody (7-B6-1) and incubated for 2 h. Then the plates were incubated with HRP-reagent and stained with TMB (Mabtech AB). Protein spots were quantified with an auto-analysis system, ImmunoSPOT S4 (Cellular Technology Ltd., Cleveland, OH, USA). Antigen-specific T-cell responses were estimated as previously described (27), and classified into four grades (+, ++, +++ and ++++) according to an algorithm described in a previous study (28). Antigen-specific T-cell responses classified (+) or more, were defined as CTL positive.

Statistical analysis. The Mann-Whitney U-test was used to compare the median values of two independent parametric continuous variables. Pearson's Chi-square or Fisher's exact test were used to compare categorical variables. OS was determined by the Kaplan-Meier analysis, with differences between survival curves assessed by the log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate analyses to identify combinations of factors that had a significant impact on survival, such as CEA, CA19-9 and CRP (14-16). All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using the overall mean values as cut-off levels. All statistical calculations were carried out using JMP® 11 (SAS Institute Inc., Cary, NC, USA). A two-sided significance level of 5% was considered statistically significant.

Results

Patients. Among the 89 patients who could be evaluated in the present study, 87 received mFOLFOX6 and two received XELOX. None of the patients was treated with bevacizumab. The peptide vaccination was administered to all patients. Forty-eight patients had at least one allele of HLA-A*24:02 and 41 had no HLA-A*24:02 allele. The characteristics of the 89 patients are summarized in Table I. The baseline characteristics were generally well balanced between the HLA-matched and HLA-unmatched groups. There was no significant difference in the incidence of injection-site reaction in the two
immunohistocytologic groups. The overall response rate was 66.7 and 58.5% in the HLA-matched and HLA-unmatched groups, respectively.

**Immune responses to the peptides.** Both IgG and T-cell responses specific to the vaccine peptides were analyzed using plasma samples obtained from the 89 patients both before and after the vaccination. For monitoring humoral responses, the levels of peptide-specific IgGs reactive to each of the five different peptides were determined by bead-based multiplex assay. Although there was a difference in degree, the values of five peptide-specific IgGs were significantly increased compared to the levels before the vaccination (Table II). The IgG response to the TOMM34 peptide was significantly higher in the HLA-A*24:02-unmatched group than the matched group, although the biological significance of this is unclear. There were no significant differences in the IgG positive rates between the HLA-matched and HLA-unmatched groups for the other peptides (Table III).

T-cell responses to the vaccine peptides were also determined by ELISpot. The CTL-positive rates for VEGFR1 and

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**Table II. Changes of peptide-specific IgG values before and after the vaccinations.**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Before vaccination</th>
<th>After vaccination</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNF43, median (IQR)</td>
<td>16.3 (0-36.3)</td>
<td>110 (18.8-1056.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TOMM34, median (IQR)</td>
<td>19.5 (0-37.6)</td>
<td>54.8 (19.3-1145)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KOC1, median (IQR)</td>
<td>14 (0-29.6)</td>
<td>19.8 (0-60.9)</td>
<td>0.038</td>
</tr>
<tr>
<td>VEGFR1, median (IQR)</td>
<td>0</td>
<td>0 (0-15.1)</td>
<td>0.014</td>
</tr>
<tr>
<td>VEGFR2, median (IQR)</td>
<td>17.5 (0-37.8)</td>
<td>58.5 (21.3-1522.1)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

IgG, immunoglobulin G; RNF43, ring finger protein 43; TOMM34, translocase of outer mitochondrial membrane 34; KOC1, kinase of the outer chloroplast membrane 1; VEGFR, vascular endothelial growth factor receptor; HLA, human leukocyte antigen; IQR, interquartile range; IgG, immunoglobulin G. aMann-Whitney U test.
VEGFR2 were significantly higher in the HLA-matched group than the HLA-unmatched group and exceeded 50% (Table III). Peptides with higher binding affinity to HLA-A*24:02 tended to be associated with higher CTL responses. Overall, there was no evident correlation between positive IgG responses and CTL responses in this analysis (Table III).

Immune responses to the peptides and clinical outcome. We also investigated whether IgG or CTL responses correlated with patient survival. First, the relationship between the IgG responses to three of the peptides (TOMM34, RNF43 and VEGFR2) and OS in HLA-matched and HLA-unmatched groups was investigated. In the HLA-matched group, OS in patients with increased IgG responses to VEGFR2 were significantly longer ($P=0.0339$) than for those with no IgG response (Fig. 1C). Conversely, there was no difference in OS for patients with increased IgG response to VEGFR2 in the HLA-unmatched group (Fig. 1C). There were no differences in OS based on IgG responses to TOMM34 and RNF43 in either the HLA-matched or -unmatched groups (Fig. 1A and B). In regard to CTLs, univariate analysis revealed no significant association of CTL induction (in response to any of the peptides) with OS (Table IV).

Multivariate analysis of prognostic factors. We also performed multivariate analysis by Cox proportional hazards regression to assess the significance of the conventional prognostic factors CEA, CA19-9 and CRP levels as well as the IgG response to VEGFR2 which was also included as covariate. This analysis demonstrated that the IgG response to VEGFR2 was the most significant predictor for OS in the HLA-A*24:02-matched group [$P=0.04$; HR, 1.99; 95% confidence interval (CI), 1.04-4.08; Table V].

### Table III. CTL induction and IgG production for five peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>A*24:02 binding affinity</th>
<th>CTL positive rate (%)</th>
<th>IgG positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All matched</td>
<td>Unmatched</td>
</tr>
<tr>
<td>RNF43</td>
<td>-6.28</td>
<td>29/79</td>
<td>15/38</td>
</tr>
<tr>
<td>TOMM34</td>
<td>-3.76</td>
<td>20/79</td>
<td>8/41</td>
</tr>
<tr>
<td>KOC1</td>
<td>-5.85</td>
<td>28/80</td>
<td>14/42</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>-7.82</td>
<td>45/83</td>
<td>28/44</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>-7.17</td>
<td>45/84</td>
<td>31/44</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fisher’s exact test.

### Table IV. Univariate analysis of CTL induction of five peptides for overall survival using Cox regression model.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>HLA-A*2402</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Matched (n=48)</td>
</tr>
<tr>
<td></td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>RNF43</td>
<td>1.08</td>
</tr>
<tr>
<td>TOMM34</td>
<td>1.11</td>
</tr>
<tr>
<td>KOC1</td>
<td>0.85</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>0.93</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1.14</td>
</tr>
</tbody>
</table>

<sup>a</sup>CTL, cytotoxic T lymphocyte; RNF43, ring finger protein 43; TOMM34, translocase of outer mitochondrial membrane 34; KOC1, kinase of the outer chloroplast membrane 1; VEGFR, vascular endothelial growth factor receptor; HLA, human leukocyte antigen; CI, confidence interval.
Table V. Univariate and multivariate analysis of biomarkers for OS using Cox regression model.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>1.23</td>
<td>0.63-2.56</td>
<td>0.55</td>
</tr>
<tr>
<td>CA19-9</td>
<td>1.12</td>
<td>0.54-2.19</td>
<td>0.76</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>1.59</td>
<td>0.72-3.28</td>
<td>0.24</td>
</tr>
<tr>
<td>VEGFR2_IgG</td>
<td>2.31</td>
<td>1.09-5.45</td>
<td>0.03</td>
</tr>
</tbody>
</table>

OS, overall survival; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CRP, C-reactive protein; VEGFR2, vascular endothelial growth factor receptor 2; CI, confidence interval; ULN, upper normal of limit.

Discussion

Recent developments in tumor immunology have led to the identification of numerous antigens and their epitope-peptides that are recognized by tumor-reactive and MHC class I-restricted CTLs (33). Cancer vaccines are acknowledged as a reliable treatment (34), however their clinical benefits have been limited (35). Therefore, to overcome this problem, it is necessary to investigate biomarkers for estimating the clinical responses to immunotherapy.

In the present study, we used HLA-A*24:02-restricted peptides and thus considered patients with HLA-A*24:02 alleles as the treatment group and patients without HLA-A*24:02 alleles as the control group, analyzing the relationship between OS and several biomarkers, including CTL and IgG responses to the vaccinated peptides. We found that the plasma levels of TOMM34 IgG, RNF43 IgG and VEGFR2 IgG significantly increased following vaccination and that increased VEGFR2 IgG responses correlated well with OS in the HLA-A*24:02-matched group. Furthermore, multivariate analysis indicated that IgG response to VEGFR2 was the most significant predictor for OS in the HLA-A*24:02-matched group. Conversely, the present study had a limitation of relevant across racial/ethnic groups. The frequency of HLA types varies greatly for each human race. HLA-A*24:02 alleles are the most frequent in Asian-Pacific Islander group including Japan, but are ranked 4th in the European group and 15th in the Africa-American group (36).

Most current peptide-based cancer vaccines have focused on cellular immune responses only, although it is well acknowledged that both cellular and humoral immune responses are important to prompt antitumor immunity in animal models (37,38). Several studies have reported that IgG responses may be a remarkable biomarker for predicting OS for vaccinated patients, although CTL responses have also shown a prognostic correlation (20,21). Previous studies recently devised a new regimen of peptide-based vaccination that consists of determining IgGs responses to various vaccine candidates, prior to administration (39-41). However, the biological roles of IgGs specific to CTL epitope peptides are practically unknown. CD4+ helper T-cells may recognize administrated peptides presented on the MHC class II molecules of dendritic cells, resulting in both the activation of helper T-cells and IgG production. It is well known that CD4+ helper T-cells are necessary to preserve CD8+ T-cell immunity (42). If increased levels of peptide-specific IgG reflect activation levels of CD4+ helper T-cells, assessment of peptide-specific IgG may be useful as an immunological biomarker to predict the clinical benefit of cancer patients undergoing peptide vaccination.

CTL responses to VEGFR1 and VEGFR2 were significantly higher in HLA-matched patients in the present study. However, the OS of patients with increased CTL responses to any of the peptides was not different to those with no CTL responses. Certainly, cellular immune responses ought to represent important clinical biomarkers, if suitable assay conditions were performed. However, currently available assays for quantifying and characterizing antigen-specific T-cell responses such as ELISpot, ELISA and 51Cr release assay have insufficient sensitivity and reproducibility for monitoring immune responses, due to lack of universal standards (28,43,44).

The VEGFR2 IgG response appeared to play a key role in improving OS in the present study. A possible explanation of this phenomenon is based on the restraint of the tumor vasculature which is crucial in immunotherapy associated with T-cell activity (45). Several clinical studies have reported a positive relation between patient survival and the presence of tumor infiltrating lymphocytes (TILs) (46). Currently, it has been recognized that the tumor vasculature organizes an important barrier against T-cells (46). Endothelial cells lining the blood vessels can downregulate T-cell activity and prevent T-cells from gaining entry into the tumor through the deregulation of adhesion molecules (47). Therefore, for clinical success, it is important to target the tumor-endothelial barrier in order to enhance T-cell activity. In conclusion, the present study revealed that the VEGFR2 IgG response may be an important immunological biomarker in patients with advanced CRC treated with a combination therapy consisting of five therapeutic epitope peptides and oxaliplatin-based chemotherapy. The biological role of IgGs specific to CTL epitope peptides remains to be determined.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors’ contributions

SH, IK and HN designed the study; YS, SH, HK, MN, YS, HM, YT, ST, YT, MI, KS, NS, ST, SY, FS, KO, KU, TF, YK and KI contributed to the patient recruitment and collected the data; SK performed the statistical analysis; SK, SH and HN wrote the manuscript. All the authors have read the manuscript and have approved this study.

Ethics approval and consent to participate

The study carried out in accordance with the Helsinki Declaration on experimentation on human subjects was approved by the Institutional Ethics submission and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Review Boards of Yamaguchi University (H20-102) and it was registered in the UMIN Clinical Trials Registry as UMIN000001791.

Consent for publication

Written informed consent was obtained from each patient at the time of enrollment.

Competing interests

SH and HN received research funding from NEC Corp., and Toyo Kohan Corp. The other authors declare that they have no competing interests.

References


