Clinical outcomes measures

Clinical response was assessed by comparing the longest diameter of the target lesions with the baseline measurement, based on the Response Evaluation Criteria in Solid Tumors (RECIST) guideline version 1.0, by caliper measurement of palpable lesions and ultrasound as previously described [11]. Briefly, complete response (CR) was defined by the disappearance of all target lesions; partial response (PR) by at least a 30 % decrease in the sum of diameters of the target lesions; PD by at least a 20 % increase in the sum of diameters of the target lesions; stable disease (SD) by neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD.

Biomarker assessments

The Onco*type* DX® 21-gene assay was performed on core biopsy and resection samples by Genomic Health [14].

Immunohistochemistry assays of Ki-67, ER and PgR were performed at one central location and the results assessed by three independent pathologists as described previously [11]. In brief, immunohistochemistry staining was performed using a Histofine kit (Nichirei, Tokyo, Japan). Ki-67 was stained using the following antibody dilution: 1:100 (Dako, Glostrup, Denmark), and the Ki-67 LI was obtained by counting 500–1,000 tumor cells at the sites of hot spots. Ki-67 groups were defined post hoc as <10, 10–30 and >30 %, respectively. ER and PgR immunoreactivity were scored according to Allred's procedure.

Expression of HER2 was determined by the HercepTest (Dako, Glostrup, Denmark). Positive HER2 status was defined as either 3+ or 2+ with confirmed c-erbB2 gene amplification by the fluorescence in situ hybridization (FISH) test.

Statistical analyses

Analyses of baseline markers included all patients with an evaluable RT-PCR result from core biopsies. Analyses of changes from baseline to post-treatment markers included the subset of patients with results from both core biopsies and surgical resections. Changes in continuous markers were defined as "post-treatment value–pre-treatment value". In the primary analysis, the rates of clinical response were compared between the high and low baseline RS groups using Fisher's exact test. Logistic regression models were fit to both clinical response and surgery type. Odds ratio (OR) estimates are presented with Wald $p$ values and 95 % confidence intervals (CIs). All $P$ values are two-sided. In exploratory analyses, the Spearman rank correlation coefficient (and associated 95 % CI) was calculated for the baseline continuous RS and either the post-treatment RS or baseline continuous Ki-67 as determined by immunohistochemistry. A paired $t$ test was applied to compare the baseline and post-treatment RS values. A two-sample $t$ test was used to compare the percentage reduction in tumor size between the high and low RS groups. Fisher's exact test was used to compare the conversion rate from mastectomy to BCS among risk groups.

## Results

A total of 116 patients were enrolled in JFMC34-0601 between March 2006 and December 2007, of whom 102 completed 24 weeks of neoadjuvant exemestane treatment [11]. Core biopsy and resection samples were obtained for 80 (69 %) and 77 (66 %) patients, respectively. Of the 157 samples sent for Onco*type* DX testing, two were deemed ineligible based on the blinded Genomic Health pathology review, insufficient RNA (<375 ng) was extracted from 18 samples (15 core biopsy and 3 resection samples), and standard quality metrics were not met for eight samples (all resections). This left 64 core biopsy samples, of which 52 had matching resection samples with evaluable RT-PCR results.

Baseline characteristics and clinical outcomes for the 64 patients are shown in Table 1. Forty-nine (76.6 %) patients had BCS, and 32 patients (50 %) had been candidates for BCS before the treatment. Four patients refused surgery after exemestane therapy and are treated as not BCS patients.

In the primary analysis, the clinical response rate in the low RS group (19/32, 59.4 %) was significantly higher than that in the high RS group (3/15, 20.0 %) ($P = 0.015$) (Table 2). The clinical response rate in the intermediate risk group (10/17, 58.8 %) was similar to that in the low risk group. Logistic regression revealed that the OR for clinical response between the intermediate and low RS groups was 0.977 (95 % CI 0.296–3.233, $P = 0.970$) and that the OR between the high and low RS groups was 0.171 (95 % CI 0.040–0.728, $P = 0.017$). In an exploratory analysis, the percentage reduction in tumor size determined by ultrasound was compared between the low and high RS groups. Patients in the low RS group showed an average reduction in tumor size of 31.8 % while those in the high RS group showed an average reduction of 12.5 %; this difference was significant between the groups ($P = 0.045$). The average reduction (27.6 %) in patients in the intermediate risk group was similar to that in the low risk group.

When treated as a continuous variable, the baseline RS Score was significantly associated with clinical response in a logistic regression analysis ($P = 0.042$; Table 3). There

**Table 1** Baseline patient characteristics and clinical outcomes ($n = 64$)

| Feature | n (%) |
|---|---|
| Age (years) | |
| 55–64 | 34 (53.1) |
| 65–74 | 25 (39.1) |
| 75–77 | 5 (7.8) |
| Tumor stage at baseline | |
| T2 | 62 (96.9) |
| T3 | 2 (3.1) |
| Stage | |
| IIA | 47 (73.4) |
| IIB | 15 (23.4) |
| IIIA | 2 (3.1) |
| ER by IHC (Allred score) | |
| 4 | 1 (1.6) |
| 5 | 3 (4.7) |
| 6 | 5 (7.8) |
| 7 | 14 (21.9) |
| 8 | 41 (64.1) |
| ER status by RT-PCR | |
| ER− ($\leq 6.5 C_T$) | 1 (1.5) |
| ER+ ($> 6.5 C_T$) | 63 (98.4) |
| PgR by IHC (Allred score) | |
| 0 | 4 (6.25) |
| 4 | 7 (10.94) |
| 5 | 4 (6.25) |
| 6 | 8 (12.5) |
| 8 | 12 (18.75) |
| NE | 10 (15.63) |
| PgR status by RT-PCR | |
| PgR− ($\leq 5.5$ $C_T$) | 14 (21.9) |
| PgR+ ($> 5.5$ $C_T$) | 50 (78.1) |
| HER2 by IHC/FISH | |
| Negative | 50 (78.1) |
| Positive | 2 (3.1) |
| Unknown | 12 (18.8) |
| RS risk group | |
| Low (<18) | 32 (50.0) |
| Intermediate (18–30) | 17 (26.6) |
| High (≥31) | 15 (23.4) |
| Ki-67 by IHC (%) | |
| <10 | 28 (43.8) |
| 10–30 | 23 (35.9) |
| >30 | 13 (20.3) |
| Clinical response | |
| Complete response (CR) | 0 |
| Partial response (PR) | 32 (50.0) |
| Stable disease (SD) | 24 (37.5) |
| Progressive disease (PD) | 5 (7.8) |
| NE | 3 (4.7) |
| Surgery type | |
| Breast-conserving | 49 (76.6) |
| Mastectomy | 11 (17.2) |
| No surgery | 4 (6.3) |

*ER* estrogen receptor, *IHC* immunohistochemistry, *RT* reverse transcriptase, *PgR* progesterone receptor, *NE* not evaluable, *FISH* fluorescence in situ hybridization, $C_T$ cycling threshold score, *RS* recurrence Score

was a trend between continuous baseline ER as determined by RT-PCR and clinical response ($P = 0.076$). Continuous baseline Ki-67 by IHC was not associated with clinical response ($P = 0.273$).

The associations between changes from baseline to post-treatment values of continuous markers and clinical response were examined in logistic regression analyses. Changes in the RS, ER as determined by RT-PCR, and Ki-67 as determined by IHC were not associated with clinical response ($P = 0.240$, 0.343 and 0.629, respectively).

Analysis of the RS categories and BCS is shown in Table 2. The OR for BCS between the intermediate and low RS groups was 0.336 (95 % CI 0.066–1.722, $P = 0.19$) and that between the high and low RS groups was 0.091 (95 % CI 0.019–0.432, $P = 0.003$). The logistic regression analyses of continuous baseline RS, ER by RT-PCR and Ki-67 by IHC with BCS are shown in Table 3. The continuous baseline RS was significantly associated with BCS in both the unadjusted ($p = 0.001$) and covariate-adjusted (for tumor size and PgR) ($P = 0.004$) analyses. The continuous baseline ER by RT-PCR was also significantly associated with BCS in both the unadjusted ($P = 0.001$) and covariate-adjusted ($P = 0.023$) analyses. Continuous baseline Ki-67 by IHC was significantly associated with BCS in the unadjusted analysis ($P = 0.024$) but lost its significance when adjusted for tumor size and PgR ($P = 0.060$). When both the continuous RS values and continuous Ki-67 were included in the logistic regression model for BCS, the RS retained its statistical significance ($P = 0.012$) whereas Ki-67 did not ($P = 0.868$). The conversion rate from mastectomy planned at baseline to BCS performed after the treatment was 88 % (15/17) in the low RS group, 70 % (7/10) in the intermediate RS group and 20 % (1/5) in the high RS group. The rate was significantly different among groups ($P = 0.010$).

The associations between RS and Ki-67, and their respective and joint associations with BCS were examined in exploratory analyses. Figure 1a shows a scatterplot of baseline Ki-67 as determined by IHC versus the baseline RS results. The Spearman correlation coefficient was 0.672 (95 % CI 0.506–0.785). All patients with PD had a high RS

**Table 2** Clinical response and breast-conserving surgery according to categorical baseline Recurrence Score

| RS risk group | Clinical response | | |
|---|---|---|---|
| | Proportion (response rate)[a] (%) | Odds ratio (95 % CI) | *P* value |
| Low (RS <18) | 19/32 (59.4) | 1 | n/a |
| Intermediate (RS 18–30) | 10/17 (58.8) | 0.977 (0.296, 3.233) | 0.970 |
| High (RS ≥31) | 3/15 (20.0) | 0.171 (0.040, 0.728) | 0.017 |
| **RS risk group** | **Breast-conserving surgery** | | |
| | Proportion (BCS rate) (%) | Odds ratio (95 % CI) | *P* value |
| Low (RS <18) | 29/32 (90.6) | 1 | n/a |
| Intermediate (RS 18–30) | 13/17 (76.5) | 0.336 (0.066, 1.722) | 0.19 |
| High (RS ≥31) | 7/15 (46.7) | 0.091 (0.019, 0.432) | 0.003 |

Data are presented as the number of patients with the percentage in parenthesis

*CI* confidence interval, *BCS* breast-conserving surgery, *n/a* not available

[a] Primary analysis: $P = 0.015$ by Fisher's exact test for comparison of clinical response rates between the low and high RS groups

**Table 3** Continuous baseline Recurrence Score and estrogen receptor by reverse transcriptase-PCR and Ki-67 by immunohistochemistry and clinical response and breast-conserving surgery

| Endpoint/analysis | Continuous marker | | | | | |
|---|---|---|---|---|---|---|
| | RS (50 units) | | ER by RT-PCR (log2 increase) | | Ki-67 by IHC (%) | |
| | Odds ratio (95 % CI) | *P* value | Odds ratio (95 % CI) | *P* value | Odds ratio (95 % CI) | *P* value |
| Clinical response/unadjusted | 0.205 (0.044, 0.946) | 0.042 | 1.436 (0.963, 2.141) | 0.076 | 0.981 (0.948, 1.015) | 0.273 |
| BCS/unadjusted | 0.055 (0.009, 0.323) | 0.001 | 1.786 (1.150, 2.774) | 0.001 | 0.957 (0.921, 0.994) | 0.024 |
| BCS/covariate-adjusted[a] | 0.016 (<0.001, 0.259) | 0.004 | 1.881 (1.090, 3.245) | 0.023 | 0.953 (0.907, 1.002) | 0.060 |

*RT* reverse transcriptase

[a] Adjusted for tumor size and PgR Allred score, which were significantly associated with BCS in the univariable analyses

(range 32–73) while three of five PD patients had an intermediate Ki-67 LI (Fig. 1a).

No statistically significant difference was observed between baseline and post-treatment RS values ($P = 0.484$). A scatterplot is shown in Fig. 1b. The Spearman correlation analysis showed a high correlation (correlation coefficient 0.745, 95 % CI 0.592–0.846).

## Discussion

In this study, we demonstrated the predictive value of the RS results for response to neoadjuvant endocrine therapy. Among our patient cohort, those with low scores showed a better response to neoadjuvant endocrine therapy than those with high scores. Since patients with high RS results have been shown to benefit from chemotherapy, the 21-gene assay may provide additional information that could facilitate the selection of neoadjuvant treatment with endocrine therapy for cancer patients with a low RS and chemotherapy for those with a high RS.

ER Allred scores have been reported to correlate with response rates to neoadjuvant letrozole or tamoxifen. The P024 trial of neoadjuvant letrozole or tamoxifen showed that tumors with low ER Allred scores still responded to letrozole [23]. Conversely, some tumors with higher ER levels did not respond to endocrine therapy [23, 24]. Gene expression-based profiles categorize HR+, HER2− breast cancers into two subtypes: luminal-A and -B [25]. However, the classification, which is based on PAM50, has been reported not to relate to clinical response or the likelihood of BCS after neoadjuvant AI treatment [7].

In our study, the RS was the only predictive factor for clinical responses to neoadjuvant endocrine therapy and the most potent predictive factor for BCS in the covariate-adjusted analysis. These results are consistent with those from other studies which suggest that a low RS can predict benefit from endocrine therapy [22, 24]. The study by Kim et al. [24] compared the outcomes of the tamoxifen and

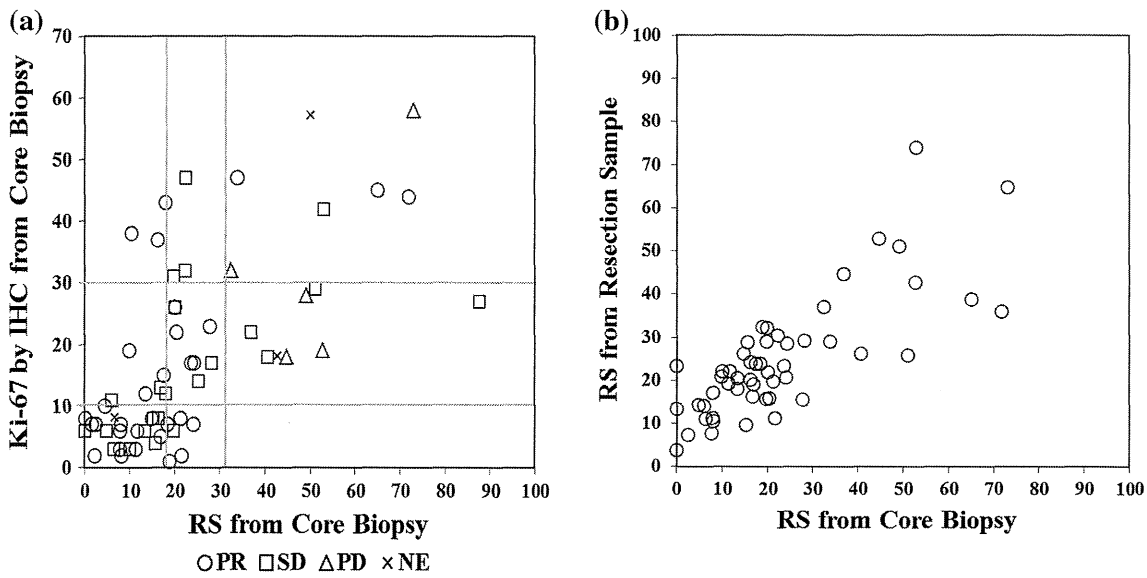


**Fig. 1** **a** Scatterplot of the baseline Recurrence score (*RS*) and baseline Ki-67, with the Spearman correlation coefficient. The Spearman correlation coefficient between the baseline RS and baseline Ki-67 was 0.672 [95 % confidence interval (CI) 0.506–0.785]. None of five patients with tumor progression was in the low or intermediate RS groups. **b** Scatterplot of the baseline RS and post-treatment RS, with the Spearman correlation coefficient. The baseline RS was highly correlated with RS in the post-treatment samples (Spearman correlation coefficient 0.745, 95 % CI 0.592–0.846). *PR* Partial response, *SD* stable disease, *PD* progressive disease, *NE* Not evaluable

placebo arms of the NSABP B14 trial and demonstrated that higher levels of quantitative ER expression, as determined by RT-PCR, correlated with a greater benefit from adjuvant tamoxifen, as measured by distant recurrence.

Our results indicate that the values of the RS before and after endocrine therapy were highly correlated. Since a number of studies have suggested that post-treatment biomarkers such as Ki-67 LI and ER have better prognostic values than pre-treatment biomarkers, post-treatment biomarkers are receiving increasing interest in clinical trials as a tool for patient stratification [26–28]. Dowsett et al. [26] reported the results of an unplanned, exploratory investigation of the relationship between post-treatment Ki-67 (2 weeks) and recurrence-free survival (RFS) using archived tumors from the IMPACT study. Their results indicate that post-treatment Ki-67, larger baseline tumor size and post-treatment ER level are significantly correlated with DFS. Ellis et al. [27] analyzed the ability of post-treatment Ki-67 and other factors (tumor size, grade, nodal status, and post-treatment ER expression) to predict RFS and breast cancer-specific survival using archived tumors from the P024 study. Another interesting study (ACOSOG Z1031, Cohort B) has been conducted to determine whether patients with a high Ki-67 value after 2 weeks of neoadjuvant AI treatment show a higher than expected pathogenic CR rate to neoadjuvant chemotherapy than would be typically observed for those patients with unselected ER-rich tumors. The results will tell us whether an assessment of Ki-67 2 weeks after neoadjuvant AI treatment will be useful for the identification of a chemotherapy-sensitive subgroup of ER+ tumors. However, even if this is the case, intervention of a 2-week AI treatment and re-biopsy are necessary. Although further investigations are needed, the comparative stability of the RS would improve the overall decision-making process regarding the complete treatment before the initiation of treatment.

The main limitation of this was its small sample size. The availability of tumor samples from the parent study was limited and recovery of mRNA was not uniformly adequate. Further investigation in larger prospective studies would better define candidates for neoadjuvant endocrine therapy. Another limitation was the absence of any assessment of lymph node response. Although nodal response is clinically relevant, one of the major purposes of neoadjuvant endocrine therapy is improvement in surgical outcome. That said, however, the clinical response at the primary site and the BCS rate are also of clinical importance for the assessment of the effect of neoadjuvant endocrine therapy.

In conclusion, this study showed that RS results have predictive value for the clinical response to neoadjuvant exemestane therapy. The 21-gene assay would appear to be a promising tool for providing useful information to guide the clinician in choosing neoadjuvant treatment for systemic therapy, with neoadjuvant endocrine treatment for patients with low RS disease and neoadjuvant chemotherapy treatment for patients with high RS disease.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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# Phase II clinical trial of S-1 plus oral leucovorin in previously treated patients with non-small-cell lung cancer☆

T. Naito [a,*], T. Seto [b], K. Takeda [c], K. Goto [d], I. Okamoto [e], K. Nakagawa [f], T. Ohba [b], H. Murakami [a], T. Takahashi [a], T. Yamanaka [g], N. Yamamoto [h]

[a] Division of Thoracic Oncology, Shizuoka Cancer Center, Japan
[b] Department of Thoracic Oncology, National Kyushu Cancer Center, Japan
[c] Department of Clinical Oncology, Osaka City General Hospital, Japan
[d] Division of Thoracic Oncology, National Cancer Center Hospital East, Japan
[e] Center for Clinical and Translational Research, Kyushu University Hospital, Japan
[f] Department of Medical Oncology, Kinki University School of Medicine, Japan
[g] Department of Biostatistics, Yokohama City University, Japan
[h] Third Department of Internal Medicine, Wakayama Medical University, Japan

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ABSTRACT

*Background:* S-1, a novel oral fluoropyrimidine, has potent antitumor activity against non-small-cell lung cancer (NSCLC). Meanwhile, leucovorin enhances the efficacy of 5-fluorouracil by inhibiting thymidylate synthase. Therefore, this phase II clinical trial evaluated the safety and efficacy of S-1 plus leucovorin combination therapy for previously treated patients with NSCLC.
*Patients and methods:* Patients with stage IIIB or IV NSCLC were prospectively enrolled if they received 1 or 2 prior chemotherapy regimens. S-1 (40–60 mg) and leucovorin (25 mg) were administered together orally twice per day for 7 consecutive days followed by 7 days of rest. This 2-week cycle was repeated for a maximum of 25 cycles until the onset of disease progression or unacceptable adverse events. Endpoints included objective tumor response, progression-free survival, overall survival, and safety.
*Results:* Among 33 patients, 6 (18.2%), 14 (42.4%), and 11 (33.3%) had partial response, stable disease, and progressive disease, respectively. Median progression-free and overall survival times were 3.5 and 11.7 months, respectively. The common grade 3 toxicities included stomatitis (18.2%), anorexia (12.1%), and neutropenia (9.1%). One patient had pneumatosis cystoides intestinalis, and another experienced paralytic ileus. There were no treatment-related deaths.
*Conclusions:* S-1 plus leucovorin combination therapy demonstrated promising efficacy and an acceptable toxicity profile in previously treated patients with NSCLC.


## 1. Introduction

Lung cancer is one of the leading causes of death worldwide [1]. Approximately 80% of lung cancers result from non-small-cell histology, and most patients present with locally advanced stage III or metastatic stage IV disease at diagnosis. Advanced non-small-cell lung cancer (NSCLC) generally results in poor outcomes, except for a small patient population with specific genetic alterations conferring susceptibility to specific molecular targeted treatments [2]. The results of phase III trials for previously treated patients with NSCLC indicate that single-agent chemotherapy with docetaxel, pemetrexed, or erlotinib as the standard chemotherapy regimen for recurrent NSCLC results in a response rate of 8.8–9.1%, median survival time of 6.7–8.3 months, and 1-year survival rate of 30–31% [3,4]. S-1 (Taiho Pharmaceutical Co. Ltd., Tokyo, Japan) is a capsule preparation comprising tegafur, an oral 5-fluorouracil (5-FU) pro-drug, 5-chloro-2,4-dihydroxypyridine (CDHP), and oteracil potassium at a molar ratio of 1.0:0.4:1.0. CDHP is a reversible competitive inhibitor of dihydropyrimidine dehydrogenase, an enzyme for 5-FU degradation. Meanwhile, oteracil potassium is a reversible competitive inhibitor of orotate phosphoribosyl transferase, an enzyme for 5-FU phosphoribosylation in the gastrointestinal mucosa [5]. The antitumor activity of S-1 against

☆ **Clinical Trials Registry Number**: UMIN000004568.
* Corresponding author at: Division of Thoracic Oncology, Shizuoka Cancer Center, 1007 Shimonagakubo, Nagaizumi-cho, Sunto-gun, Shizuoka, Japan.
Tel.: +81 55 989 5222; fax: +81 55 989 5634.
*E-mail address:* t.naito@scchr.jp (T. Naito).

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NSCLC has been proven in several clinical trials. First-line treatment of S-1 combined with platinum showed favorable outcomes in 2 phase III trials for metastatic NSCLC [6,7]. Chemoradiation with S-1 plus cisplatin also showed promising results in locally advanced NSCLC [8,9]. In second- or third-line settings, several phase II trials demonstrate promising antitumor activity of S-1 monotherapy for previously treated patients with advanced NSCLC [10–13]. The addition of leucovorin increases the intracellular concentration of reduced folates, thus stabilizing the 5-fluorodeoxyuridine monophosphate/thymidylate synthase enzyme complex, providing the biochemical rationale for adding leucovorin to 5-FU and tegafur chemotherapy regimens [14,15]. An in vivo study of S-1 plus leucovorin treatment using xenograft mouse models of human colorectal cancer cells demonstrated that leucovorin might improve the antitumor activity of S-1 [16]. A phase II clinical trial of S-1 plus oral leucovorin for chemotherapy-naïve patients with metastatic colorectal cancer recently demonstrated promising efficacy [17]. In addition, this treatment might improve the convenience of cancer care because of the combination of oral medicines. Accordingly, the present phase II study evaluated the safety and efficacy of S-1 plus leucovorin combination therapy in previously treated patients with advanced NSCLC.

## 2. Methods

### 2.1. Patients

This was an open-labeled, multicenter, single-arm, phase II study. Patients were enrolled from the following 5 institutions: Kinki University, the National Cancer Center Hospital East, the National Kyushu Cancer Center, Osaka City General Hospital, and the Shizuoka Cancer Center. The eligibility criteria were as follows: (1) histologically and/or cytologically proven stage IIIB or IV NSCLC with at least 1 measurable lesion; (2) 1 or 2 previous cytotoxic chemotherapy regimens; *EGFR* tyrosine kinase inhibitors and adjuvant chemotherapy were not counted as a prior treatment; and (3) Eastern Cooperative Oncology Group performance status 0–1 and adequate organ function. Patients were excluded if they had received systemic chemotherapy or thoracic radiation within the previous 4 weeks, radiation to extrathoracic lesions within the previous 2 weeks, or previous treatment with fluoropyrimidine agents. Patients with serious medical conditions including other malignancies, symptomatic brain metastases, psychiatric disorders, active infectious diseases, and active ischemic heart disease were also excluded. A data and safety monitoring board monitored the trial on an ongoing basis. The protocol, protocol amendments, informed consent, and other documents pertaining to the study were approved by the institutional review board of each participating center. The first and last authors vouch for the accuracy and completeness of the data and analyses reported as well as the fidelity of the report to the study protocol. This trial is registered on the clinical trials site of the University Hospital Medical Information Network Clinical Trials Registry in Japan (registration number: UMIN000004568).

### 2.2. Treatment plan

The dose of S-1 (capsules containing tegafur 20 or 25 mg) was determined according to body surface area as follows: 40, 50, and 60 mg for <1.25, 1.25–1.50, and ≥1.50 $m^2$, respectively.

Leucovorin (25-mg tablets) was administered at a fixed dose of 25 mg. S-1 and leucovorin were administered together orally twice per day for 7 consecutive days followed by 7 days of rest; this 2-week cycle was repeated for a maximum of 25 cycles until the onset of disease progression or unacceptable adverse events.

**Table 1**
Patient characteristics.

| Characteristics | N = 33 | % |
|---|---|---|
| Gender (male:female) | 25:8 | |
| Age, median (range) | 65 (27–74) | |
| ECOG-PS 0 | 13 | 39.4 |
| 1 | 20 | 60.6 |
| Histology | | |
| Adenocarcinoma | 26 | 78.8 |
| Squamous cell carcinoma | 4 | 12.1 |
| Large cell carcinoma | 2 | 6.1 |
| Pleomorphic carcinoma | 1 | 3.0 |
| Stage | | |
| IIIB | 5 | 15.2 |
| IV | 28 | 84.8 |
| No. of prior chemotherapy | | |
| 1 Regimen | 11 | 33.3 |
| 2 Regimens | 19 | 57.6 |
| 3 Regimens | 3 | 9.1 |

The dose of S-1 could be decreased by 2 levels to a minimum dose of 20 mg twice daily in the event of following toxicities: grade 4 neutropenia or non-hematologic toxicity, or grade 3 thrombocytopenia, diarrhea, stomatitis, or skin rash. The dose of leucovorin was not decreased.

### 2.3. Study assessment

Tumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors version 1.1, and computed tomography scans were performed every 4–6 weeks. If a patient responded, response was confirmed through tumor assessments at least 4 weeks after the first documentation of a response. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. Physical examination, chest radiograph, laboratory chemistry, and hematology were performed at baseline and on day 1 of each cycle.

### 2.4. Statistical analysis

The primary endpoint of the study was the antitumor activity of S-1 plus leucovorin assessed according to the overall response rate (ORR) including complete response (CR) and partial response (PR). The secondary endpoints were overall survival (OS), progression-free survival (PFS), and safety profile. We defined acceptable and unacceptable ORRs as 20% and 5%, respectively. The sample size was determined to be 30 on the basis of the exact binomial probability distribution of Southwest Oncology Group 2-stage design with a statistical power ($1-\beta$) of 80% and significance level ($\alpha$) of 5%. All analyses were performed using JMP version 9.0 for Windows (SAS Institute Inc., Cary, NC, USA).

## 3. Results

### 3.1. Patient characteristics

From December 2010 through September 2011, a total of 33 patients (median age: 65 years, range: 27–74 years) who met the inclusion criteria were enrolled (Table 1). The majority of the patients had stage IV disease (28 patients, 84.8%), including 5 patients (15.2%) with postoperative relapse. Histopathological diagnoses included adenocarcinoma, squamous-cell carcinoma, large-cell carcinoma, and pleomorphic carcinoma in 26, 4, 2, and 1 patient, respectively. An activating *EGFR* gene mutation was assessed in 26 patients, 5 of whom had a mutant gene. Regarding prior chemotherapy, 1 patient had received platinum-based chemoradiotherapy, and 2 patients had received gefitinib

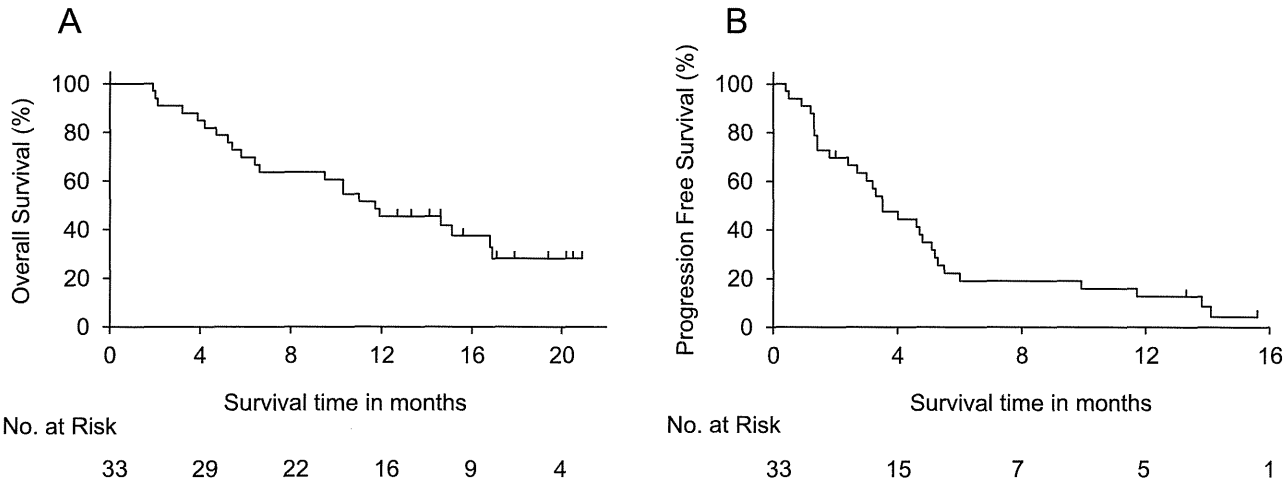


**Fig. 1.** (A) Kaplan Meier survival curve of overall survival and (B) Kaplan Meier survival curve of progression free survival.

as a first-line treatment. The remaining 30 patients had received platinum-based chemotherapy with or without bevacizumab as a first-line treatment. A total of 23 patients had received second-line or more chemotherapy before study entry.

### 3.2. *Treatment delivery*

A total of 255 treatment cycles were administered to patients. The median number of treatment courses was 6 (range: 1–25). The median treatment period was 2.5 months (95% confidential interval [CI]: 1.1–4.0 months). Dose reduction and treatment interruption were required in 13 (39.4%) and 6 (18.2%) patients, respectively. The reasons for treatment withdrawal were disease progression in 22 (66.7%), toxicities in 4 (12.1%), protocol completion in 3 (9.1%), and patient preference in 4 (12.1%). The median total doses per 6 weeks for S-1 and leucovorin were 2100 mg (range: 840–2520 mg) and 1050 mg (range: 350–1050 mg), respectively. The median relative dose intensity for the first 6 weeks for S-1 and leucovorin were 82.5% (95% CI: 74.8–90.3%) and 84.5% (95% CI: 76.8–92.2%), respectively.

### 3.3. *Efficacy*

The objective tumor response (the primary endpoint) was assessed by independent evaluators in all 33 patients. One woman was considered unevaluable for tumor response because she asked to discontinue the study treatment after 1 course because of grade 1 mucositis and declined radiological assessment. Among the remaining 32 patients, 0, 6, 15, and 11 had complete response, partial response, stable disease, and progressive disease, respectively. The response rate was 18.2% (95% CI: 7.0–35.5%), and the disease control rate was 63.6% (95% CI: 45.1–79.6%, Table S1). Although the patients had heterogeneous background characteristics including pathological diagnosis and the number of previous treatments, most patients experienced tumor shrinkage or stabilization during the study period (Fig. S1). All 33 patients were evaluable for the OS and PFS, and their median follow-up duration was 17.9 (95% CI: 14.1–20.2) months. The cutoff date for analysis was November 6, 2012. At the time of analysis, 11 (33.3%), 3 (9.1%), and 0 (0%) patients were alive, free of progression, and on study treatment, respectively. Median survival time was 11.7 months (95% CI: 6.1–16.9 months) and the 1-year survival rate was 45.5% (95% CI: 29.6–62.3%, Fig. 1A). Median PFS was 3.5 months (95% CI: 2.4–5.1 months, Fig. 1B), and the median time to treatment failure was 2.5 months (95% CI: 1.1–4.0 months). A Comparison of efficacy with S-1 monotherapy showed a relatively better efficacy profile in our study treatment (Table 2). A comparison of efficacy among histology types was also summarized in Table S2. A total of 2 out of 26 patients with adenocarcinoma (7.7%) and 4 out of 7 patients with non-adenocarcinoma (57.1%) showed partial response ($p = 0.2233$, Fisher's exact test) including 2 squamous carcinoma, 1 pleomorphic carcinoma, and 1 large cell carcinoma. Median OS was 10.3 in patients with adenocarcinoma and not reached in non-adenocarcinoma ($p = 0.0505$, log-rank test). A total of 19 patients (57.6%) received additional treatments after the study treatment, including docetaxel, erlotinib with or without investigational drugs in clinical trials, gemcitabine, pemetrexed, and palliative radiation therapy in 5, 5, 4, 2, and 3 patients, respectively.

### 3.4. *Safety and adverse events*

Safety data from all 33 patients are shown in Table 3. All toxicities with an incidence ≥50% included anemia (93.9%), hypoalbuminemia (87.9%), anorexia (84.8%), stomatitis (72.7%), fatigue (60.6%), pigmentation (57.6%), nausea (54.5%), and leukocytopenia (51.5%). Grade 3 toxicity occurred in 15 patients (45.5%). Grade 3 toxicities with an incidence ≥10% included stomatitis (18.2%) and anorexia (12.1%). One patient each had pneumatosis cystoides intestinalis (grade 3) and paralytic ileus (grade 3); both toxicities improved as a result of interrupting treatment and subsequently resuming treatment with a reduced dose. There were no grade 4 toxicities, febrile neutropenia, or interstitial lung disease. The dose was reduced at least once in 13 patients (39.4%), mainly because of stomatitis and anorexia. Rest periods were prolonged in 15 patients (45.5%), mainly because of persistent stomatitis, anorexia, and fatigue. The median number of treatment courses until the worst grade of stomatitis, anorexia, fatigue, diarrhea, and rash was 2, 1, 3, 2, and 1, respectively. There were no treatment-related deaths. A Comparison of ≥grade 3 adverse events with S-1 monotherapy showed increased percentage of anorexia, stomatitis, and neutropenia in our study treatment (Table 3).

## 4. Discussion

This multicenter phase II clinical trial demonstrates the efficacy and safety of S-1 plus oral leucovorin combination therapy for previously treated patients with NSCLC. The results show that the treatment has promising antitumor activity, with an objective response rate of 18.2%, which meets the primary endpoint of this

**Table 2**
Comparison of efficacy with S-1 monotherapy.

| Efficacy | Our study | Totani et al. [12] | Shiroyama et al. [11] | Govindan et al. [10] | Wada et al. [13] |
|---|---|---|---|---|---|
| N | 33 | 48 | 44 | 57 | 30 |
| Treatment line | 2nd or 3rd | 2nd | 2nd | 2nd | ≥2nd |
| Response rate (%) | 18.2 | 12.5 | 13.6 | 7.1 | 26.7 |
| Disease control rate (%) | 63.6 | 39.6 | 77.3 | 55.3 | 70.0 |
| Median PFS (months) | 3.5 | 2.5 | 4.2 | 2.9 | 3.1 |
| Median OS (months) | 11.7 | 8.2 | 16.4 | 7.3 | 11.2 |
| 1-year survival rate (%) | 45.5 | 29.6 | 60.3 | 31.6 | 43.3 |

PFS, progression-free survival; OS, overall survival.

study. The treatment was safe and tolerable for all patients, and there were no grade 4 toxicities or treatment-related deaths.

Leucovorin is a biochemical modulator of 5-FU that stabilizes the inhibitory ternary complex formed between thymidylate synthase and the active metabolite of 5-FU, 5-fluorodeoxyuridylate. A meta-analysis of advanced colorectal cancer cases revealed that leucovorin improves response rates and OS when combined with 5-FU in comparison to 5-FU alone [18]. The 5-FU/leucovorin-based regimens such as 5-FU/leucovorin plus oxaliplatin and/or irinotecan are standard treatments for metastatic colorectal cancer [19]. The role of S-1 in the treatment of other solid tumors including gastric, colorectal, biliary tract, pancreatic, and lung cancers has recently been increasing [20–22]. The antitumor activity of S-1 against NSCLC has been proven in several clinical trials [6–8]. There are several reports of S-1 monotherapy as a second-line or subsequent-line treatment for previously treated NSCLC [10–13], with response rates ranging from 7.1% to 26.7%, median PFS from 2.5 to 4.2 months, median survival time from 8.2 to 16.4 months, and the 1-year survival rate from 29.6% to 60.3% (Table 2). Relatively low incidences of severe toxicities (i.e., grade 3 or 4) were reported, and the treatment was considered to be well tolerated.

The present study is the first report of the efficacy and safety of S-1/leucovorin combination therapy for advanced NSCLC. The results revealed a relatively high response rate and long PFS, indicating that leucovorin potentiates the antitumor activity of S-1. However, regarding safety, the incidence of toxicity was higher with S-1/leucovorin combination therapy in the present study than with S-1 monotherapy in previous studies; approximately 45% of the present patients experienced grade 3 toxicities such as stomatitis, anorexia, and neutropenia in comparison to <20% of patients receiving S-1 monotherapy. Similarly, in the clinical trial of S-1/leucovorin combination therapy for colorectal cancer, treatment resulted in a relatively high incidence of non-hematologic toxicities. In the original 4-week regimen, in which S-1/leucovorin was administered for 2 weeks followed by 2 weeks of rest, grade 3 toxicities occurred in 55% of patients, including diarrhea, anorexia, stomatitis, and neutropenia in 32%, 21%, 20%, and 14%, respectively. As a result, 59% of the patients in that study required dose reduction, and 54% required a prolonged rest period [17]. A modified less-toxic treatment schedule in which S-1/leucovorin is administered for 1 week followed by 1 week of rest was recently proposed in a multicenter international phase II study conducted in Japan and China [23]. This regimen resulted in decreased occurrence of severe toxicities associated with this combination therapy without reducing relative dose intensity or efficacy. Grade 3 diarrhea, anorexia, stomatitis, and neutropenia occurred in 8.3%, 2.8%, 8.3%, and 9.7% of patients, respectively. Although we used the latter treatment schedule (i.e., 1 week on/1 week off), the incidences of stomatitis (18.2%) and anorexia (12.1%) were slightly higher. This might be due to the differences in patient characteristics between studies: our patients were administered 1 or more chemotherapeutic regimens, while the other study included

**Table 3**
Treatment-related adverse events.

| Adverse events, N (%)[a] | Any grade | Grade 2 | Grade 3 | Reference[b] ≥Grade 3 in S-1 monotherapy (%) |
|---|---|---|---|---|
| Non-hematologic | | | | |
| Anorexia | 28(84.8) | 15(45.5) | 4(12.1) | 2.1–7.1 |
| Stomatitis | 24(72.7) | 10(30.3) | 6(18.2) | 0.0–3.6 |
| Fatigue | 20(60.6) | 11(33.3) | 1(3.0) | 0.0–12.5 |
| Hyperpigmentation | 19(57.6) | 4(12.1) | – | – |
| Nausea | 18(54.5) | 9(27.3) | – | 0.0–5.4 |
| Vomiting | 12(36.4) | 5(15.2) | 0(0.0) | 0.0–1.8 |
| Diarrhea | 15(45.5) | 5(15.2) | 1(3.0) | 0.0–21.4 |
| Constipation | 13(39.4) | 3(9.1) | 0(0.0) | 0.0 |
| Skin rash | 13(39.4) | 5(15.2) | 1(3.0) | 1.8–2.1 |
| Alopecia | 5(15.2) | – | – | – |
| Hematologic | | | | |
| Anemia | 31(93.9) | 14(42.4) | 1(3.0) | 1.8–4.5 |
| Hypoalbuminemia | 29(87.9) | 7(21.2) | 0(0.0) | 0.0 |
| Leukocytopenia | 17(51.5) | 7(21.2) | 2(6.1) | 0.0–4.5, |
| Hyponatremia | 14(42.4) | 0(0.0) | 2(6.1) | 0.0 |
| Hypocarcemia | 13(39.4) | 2(6.1) | 0(0.0) | 0.0 |
| Neutropenia | 10(30.3) | 6(18.2) | 3(9.1) | 2.1–4.5 |
| Thrombocytopenia | 9(27.3) | 0(0.0) | 0(0.0) | 0.0 |
| Hypokalemia | 6(18.2) | 0(0.0) | 2(6.1) | 0.0 |
| Alkaline phosphatase increased | 6(18.2) | 2(6.1) | 0(0.0) | 0.0 |
| Hyperkalemia | 6(18.2) | 0(0.0) | 0(0.0) | 0.0 |
| Total bilirubin increased | 6(18.2) | 0(0.0) | 0(0.0) | 0.0 |

[a] No grade 4 or more toxicity was reported.
[b] The data was a summary of Refs. [10–13].

only chemotherapy-naïve colorectal cancer patients. In addition, the median age was higher (65 vs. 60 years) and the percentage of ECOG-PS grade 0 was lower (39.4% vs. 54.9%) in our patients than that in the previous study. However, in the present study, all of the toxicities were easily manageable by routine supportive care with short treatment interruption, and most of the patients were able to resume treatment with or without dose reduction.

A major limitation of this study is a small study population comprising exclusively Japanese patients. Accordingly, the toxicity profile of S-1 is reported to differ by ethnicity [10,24]. The primary dose-limiting toxicity of S-1 in American and European clinical trials was gastrointestinal toxicity including diarrhea and nausea/vomiting [25,26], whereas that in Japanese clinical trials was hematological toxicity [27]. Because S-1/leucovorin combination therapy resulted in a relatively high incidence of gastrointestinal toxicities, caution should be exercised when administering this treatment to patients of different ethnicities, especially American and European populations.

In conclusion, this phase II study demonstrates that S-1 with oral leucovorin combination therapy has promising antitumor activity and is well tolerated in previously treated patients with NSCLC. Nevertheless, further large-scale Phase III clinical trials comparing the efficacy of S-1/leucovorin combination therapy with current standard treatment are required to confirm the benefits of this treatment.

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## Conflict of interest statement

None declared.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2014.10.010.

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RESEARCH Open Access

# A phase II study of five peptides combination with oxaliplatin-based chemotherapy as a first-line therapy for advanced colorectal cancer (FXV study)

Shoichi Hazama[1*], Yusuke Nakamura[2], Hiroaki Tanaka[3], Kosei Hirakawa[3], Ko Tahara[4], Ryoichi Shimizu[5], Hiroaki Ozasa[5], Ryuichi Etoh[5], Fumiaki Sugiura[6], Kiyotaka Okuno[6], Takumi Furuya[7], Taku Nishimura[8], Koichiro Sakata[8], Kazuhiko Yoshimatsu[9], Hiroko Takenouchi[1], Ryouichi Tsunedomi[1], Yuka Inoue[1], Shinsuke Kanekiyo[1], Yoshitaro Shindo[1], Nobuaki Suzuki[1], Shigefumi Yoshino[1], Hirokazu Shinozaki[10], Akira Kamiya[10], Hiroyuki Furukawa[10], Takeharu Yamanaka[11], Tomonobu Fujita[12], Yutaka Kawakami[12] and Masaaki Oka[1]

## Abstract

**Background:** We previously conducted a phase I trial for advanced colorectal cancer (CRC) using five HLA-A*2402-restricted peptides, three derived from oncoantigens and two from vascular endothelial growth factor (VEGF) receptors, and confirmed safety and immunological responses. To evaluate clinical benefits of cancer vaccination treatment, we conducted a phase II trial using the same peptides in combination with oxaliplatin-based chemotherapy as a first-line therapy.

**Methods:** The primary objective of the study was the response rates (RR). Progression free survival (PFS), overall survival (OS), and immunological parameters were evaluated as secondary objective. The planned sample size was more than 40 patients for both HLA2402-matched and -unmatched groups. All patients received a cocktail of five peptides (3 mg each) mixed with 1.5 ml of IFA which was subcutaneously administered weekly for the first 12 weeks followed by biweekly administration. Presence or absence of the HLA-A*2402 genotype were used for classification of patients into two groups.

**Results:** Between February 2009 and November 2012, ninety-six chemotherapy naïve CRC patients were enrolled under the masking of their HLA-A status. Ninety-three patients received mFOLFOX6 and three received XELOX. Bevacizumab was added in five patients. RR was 62.0% and 60.9% in the HLA-A*2402-matched and -unmatched groups, respectively ($p = 0.910$). The median OS was 20.7 months in the HLA-A*2402-matched group and 24.0 months in the unmatched group (log-rank, $p = 0.489$). In subgroup with a neutrophil/lymphocyte ratio (NLR) of < 3.0, patients in the HLA-matched group did not survive significantly longer than those in the unmatched group (log-rank, $p = 0.289$) but showed a delayed response.

**Conclusions:** Although no significance was observed for planned statistical efficacy endpoints, a delayed response was observed in subgroup with a NLR of < 3.0. Biomarkers such as NLR might be useful for selecting patients with a better treatment outcome by the vaccination.

**Trial registration:** Trial registration: UMIN000001791.

**Keywords:** Peptide vaccine, Peptide cocktail, Colorectal cancer, Phase II study, FOLFOX, Chemotherapy

* Correspondence: hazama@yamaguchi-u.ac.jp
[1]Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Ube, Japan
Full list of author information is available at the end of the article

## Background

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related death in industrialized countries [1]. In the past decade, a combination treatment of fluorinated-pyrimidine with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX, XELOX), with or without monoclonal antibodies such as anti-vascular endothelial growth factor (VEGF) antibody or anti-epidermal growth factor receptor (EGFR) antibody, has markedly improved the prognosis of patients with metastatic CRC (mCRC) [2-6]. However, most of the patients reveal progression of the disease due to chemo-resistance and lose their lives.

As an attempt to validate a new treatment modality to overcome the limited disease control status of mCRC, we conducted a combination treatment of five therapeutic epitope-peptides with chemotherapy. Recent developments in genome-based technologies have enabled us to obtain comprehensive gene expression profiles of malignant cells and compare them with normal cells [7]. We had previously identified three oncoantigens, RNF43 (ring finger protein 43) [8], 34 kDa translocase of the outer mitochondrial membrane (TOMM34) [9], and KOC1 (IMP-3; IGF-II mRNA binding protein 3) [10], as targets for the development of cancer peptide vaccines for CRC.

Although immunotherapy using tumor infiltrating cells (TIL) or vaccine treatment are promising modalities for the treatment of cancer, recent reports have indicated several mechanisms in tumor tissues which make cancer cells escape from immune system attacks [11]. For example, the limited antitumor effects of cytotoxic T lymphocytes (CTL) were explained by tumor heterogeneity; a subset of tumor cells revealed the down-regulation or absence of human leukocyte antigen (HLA) or targeted antigen proteins [12,13]. Since the growth of solid neoplasms is almost always accompanied with neovascularization [14], which is associated with the expression of vascular endothelial growth factor receptor 1 (VEGFR1) [15] and/or VEGFR2 [16], our vaccine treatment also included the peptides derived from VEGFR1 and VEGFR2 that target neovascular endothelial cells. We selected five HLA-A*2402-restricted peptides derived from RNF43, TOMM34, KOC1, VEGFR1, and VEGFR2 for the clinical trial due to the abundance of the HLA-A*2402 allele in the Japanese population (an allelic frequency of approximately 60%) [17]. We previously performed a phase I study of a combination vaccine treatment for mCRC, and confirmed the safety and the promising potential of our five-peptide-cocktail treatment to improve the prognosis of advanced CRC [18].

FOLFOX (or XELOX) with/without bevacizumab is a widely-used chemotherapy [4] and has been reported to possibly reduce the number of Tregs [19]. We therefore conducted a phase II study of a cancer vaccine consisting of five peptides in combination with oxaliplatin-based chemotherapy as a first-line therapy for advanced CRC.

The purpose of this study was to evaluate the clinical benefit of this cancer vaccine treatment by adding to oxaliplatin-based chemotherapy. Furthermore, we explored a predictive biomarker for its response and for the selection of patients who are likely to exhibit better treatment outcomes following the vaccine treatment. We here demonstrate a promising result of our combination immuno-chemotherapy and predictive biomarkers for immunotherapy.

## Patients and methods

### Patients and eligibility criteria

Patients were eligible for enrollment when they were ≥ 20 years old with a histologically confirmed advanced CRC, had one or more measurable lesions according to the Response Evaluation Criteria in Solid Tumors version 1.0 (RECIST), were naïve for chemotherapy, had adequate functions of critical organs, had an ECOG performance status (PS) of 0 or 1, and had a life expectancy of ≥ 3 months. The exclusion criteria were CNS involvement, second primary tumors, active infectious disease, any steroid treatment, or any prior peptide vaccination therapies. Written informed consent was obtained from each patient at the time of enrollment. The study was carried out in accordance with the Helsinki declaration on experimentation on 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 as UMIN000001791.

### Peptides

The RNF43-721 (NSQPVWLCL) [20], TOMM34-299 (KLRQEVKQNL) [9], KOC1(IMP-3)-508 (KTVNELQNL) [21], VEGFR1-1084 (SYGVLLWEI) [22] and VEGFR2-169 (RFVPDGNRI) [23] peptides restricted with HLA-A*2402 were synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA) according to a standard solid-phase synthesis method, and were purified by reverse-phase high performance liquid chromatography (HPLC). The purity (> 95%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. Endotoxin levels and the bio-burden of these peptides were tested and determined to be within acceptable levels as Good Manufacturing Practice grade for vaccines.

### Study design

This phase II, single arm, non-randomized, HLA-A status double-blind study was conducted to assess the efficacy of this combination therapy for first-line treatment for advanced CRC. 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 HLA-A*2402 restricted peptides, all enrolled patients whose HLA-A status were double-blinded were administrated the same regime of peptide cocktail and oxaliplatin-containing chemotherapy.

The cocktail of 3 mg each of five peptides derived from RNF43-721, TOMM34-299, KOC1-508, VEGFR1-1084 and VEGFR2-169, 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 regions on day 1 of each week for 13 weeks, then the vaccination schedule was reduced to once every 2 weeks. Vaccination was continued even if the disease progressed when the patient wished and a primary doctor who provided additional chemotherapies agreed.

Oxaliplatin-containing regimens were administrated concurrently with the vaccination. Detailed informations of the chemotherapies were described in Additional file 1. Briefly, mFOLFOX6 [24,25] consisted of oxaliplatin (85 mg/m$^2$) with leucovorin (400 mg/m$^2$), followed by a FU (400 mg/m$^2$) bolus, and then 2,400 mg/m$^2$ continuous infusion with/without bevacizumab (5 mg/kg) [4]. This treatment was repeated every 14 days. XELOX [4] consisted of oxaliplatin (130 mg/m$^2$) on day 1 followed by oral capecitabine (1,000 mg/m$^2$) twice daily on days 1 through 14 of a 21-day cycle with/without bevacizumab at a dose of 7.5 mg/kg.

### Study objectives

The primary objective was the comparison of the efficacy of the peptide-cocktail plus oxaliplatin-containing regimen on patients with HLA-A*2402 compared with those without HLA-A*2402 by assessing the objective response rate (ORR; complete response (CR) and partial response (PR)). Secondary objectives included comparisons between the two groups for progression free survival (PFS), overall survival (OS), safety, and tolerability. Exploratory end points included the assessments of tumor and blood-based immunological biomarkers.

### Assessments

Medical history, physical examination, chest X-ray, ECG, and carcinoembryonic antigen (CEA) measurements were performed within 21 days before starting the treatment. Assessments of vital signs, ECOG performance status, height, weight, and routine blood analysis (hematology and chemistry) were performed within 7 days of starting the treatment. During treatment, physical examination, hematology, and biochemistry analyses were repeated on day 1 of every treatment cycle. Tumor assessments (computed tomography scan, magnetic resonance imaging) were made before starting the study treatment and were repeated every 4 to 8 weeks after the treatment. The RECIST guidelines were used to define all responses. Signs of hematological toxicity and non-hematological toxicity were assessed according to CTCAE during therapy and for 28 days after the last study drug dose.

### Immunological biomarkers

We investigated the neutrophil/lymphocyte ratio (NLR) and the peripheral blood lymphocyte counts per the entire white blood cells (lymphocyte-%) before the treatment as predictive markers of the efficacy of the vaccination. NLR and lymphocyte-% were determined immediately at each study site.

### Statistical analysis

This study was designed to test the hypothesis that a regime consisting of vaccination plus oxaliplatin-containing

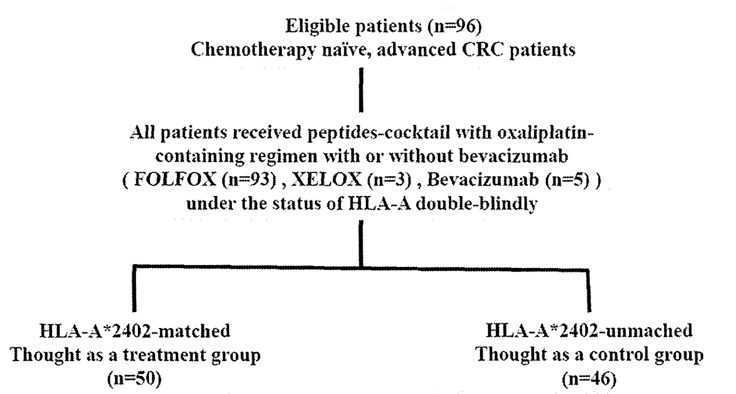


**Figure 1 CONSORT diagram.** Scheme showing an HLA-A-status double-blind, biologically-randomized phase II study of five therapeutic epitope-peptides combined with oxaliplatin-based chemotherapy as a first-line therapy for advanced colorectal cancer (FXV study). CRC, colorectal cancer; FOLFOX, infusional fluorouracil, leucovorin, and oxaliplatin; XELOX, capecitabine and oxaliplatin; HLA, human leukocyte antigen.

**Table 1 Baseline Patient Characteristics**

| Characteristics | | HLA-A*2402 | | p value |
|---|---|---|---|---|
| | | Matched (n = 50) | Unmatched (n = 46) | |
| Sex | | | | |
| | Male | 25 | 24 | NS |
| | Female | 25 | 22 | |
| Age | | | | |
| | Mean | 64.3 | 63.4 | NS |
| | Standard error | 10.9 | 8 | |
| | Range | 36-82 | 38-77 | |
| Unresectable site | | | | |
| | Liver | 27 | 35 | |
| | Lung | 18 | 12 | |
| | Dissemination | 5 | 4 | NS |
| | Bone | 1 | 2 | |
| | Lymphnode | 13 | 13 | |
| | Other | 5 | 1 | |
| Number of unresectable sites | | | | |
| | 1 | 36 | 30 | |
| | 2 | 9 | 11 | |
| | 3 | 5 | 5 | |
| Resection of primary lesion | | | | |
| | yes | 41 | 43 | |
| | no | 9 | 3 | NS |
| Chemotherapy | | | | |
| | FOLFOX | 48 | 45 | |
| | XELOX | 2 | 1 | NS |
| | (Bevacizumab) | 0 | (5) | |
| Primary minor site | | | | |
| | Colon | 29 | 36 | 0.057 |
| | Rectal | 21 | 10 | |

FOLFOX. infusional fluorouracil. leucovorin. and oxaliplatin: XELOX. capecitabine and oxaliplatin; HLA, human leukocyte antigen; NS. not significant.

chemotherapy is more effective for patients with HLA-A*2402 positive aCRC when compared to those without HLA-A*2402, defining the HLA-A*2402 matched group as the study group and the unmatched group as the control group. Because the response rate of colorectal cancer patients to first line-treatment is generally about 50%, we estimated that a minimum of 40 patients for both arms would be required, assuming a response rate of 50% in the HLA-unmatched control group and 65% in the HLA-matched study group. A two-sided Alpha level of 0.2 and a beta level of 0.5 were assumed.

Response rates were compared by chi-squared test. OS and PFS rates were analyzed by the Kaplan-Meier method and log rank test. For the evaluation of delayed response, we also performed a supplemental analysis of the weighted log-rank tests with the Harrington-Fleming class of weights test for 3 parameter settings ($\rho = 0$ and $\gamma = 0.5$; $\rho = 0$ and $\gamma = 1$; $\rho = 0$ and $\gamma = 2$) [26].

Statistical analyses were performed using SPSS statistics version 20 (SPSS, Chicago, IL, USA) and SAS v9.2. A p value < 0.05 was considered statistically significant.

## Results

### Patients

Between January 2009 and November 2012, ninety-six patients were enrolled in this trial applying the peptide cocktail treatment in combination with an oxaliplatin-based regimen in 13 hospitals. Fifty patients had at least one allele of HLA-A*2402 and forty-six patients had no HLA-A*2402 allele. The peptide vaccination was administered to all patients. Among the 96 patients enrolled to this trial, 93 patients received mFOLFOX6 and three received XELOX. Five patients were additionally treated with bevacizumab (Figure 1). The baseline characteristics were generally well balanced between the HLA-matched and HLA-unmatched groups, although the proportion of rectal cancer was slightly higher in the HLA-matched group (Table 1). On the cut-off date (25 December, 2013), 87 patients (91%) revealed the progression of the disease with the median OS follow-up period of 38.2 months.

### Objective response rate

The ORR was 62.0% and 60.9% in the HLA-matched and HLA-unmatched groups (p = 0.910), respectively (Table 2). The proportions of CR, PR, and SD as well as the disease control rate were 2.0% (1/50), 60.0% (30/50), 32.0% (16/50), and 94.0% (47/50) in the HLA-matched group, respectively, and 0% (0/46), 60.9% (28/46), 37.0% (17/46), 97.8% (45/46) in the HLA-unmatched group, respectively.

### Progression free survival

The median PFS was 7.2 months for the HLA-matched group and 8.7 months for the HLA-unmatched group. There was no significant difference between two groups (Figure 2A, P = 0.971). We also performed sub-group analyses using

**Table 2 Objective Response rate**

| HLA-status | HLA-A*2402-matched | | | (n = 50) | HLA-A*2402-unmatched | | | (n = 46) |
|---|---|---|---|---|---|---|---|---|
| Response | CR | PR | SD | PD | CR | PR | SD | PD |
| Number | 1 | 30 | 16 | 3 | 0 | 28 | 17 | 1 |
| Response rate | | 31/50 | (62.0%) | | | 28/46 | (60.9%) | |

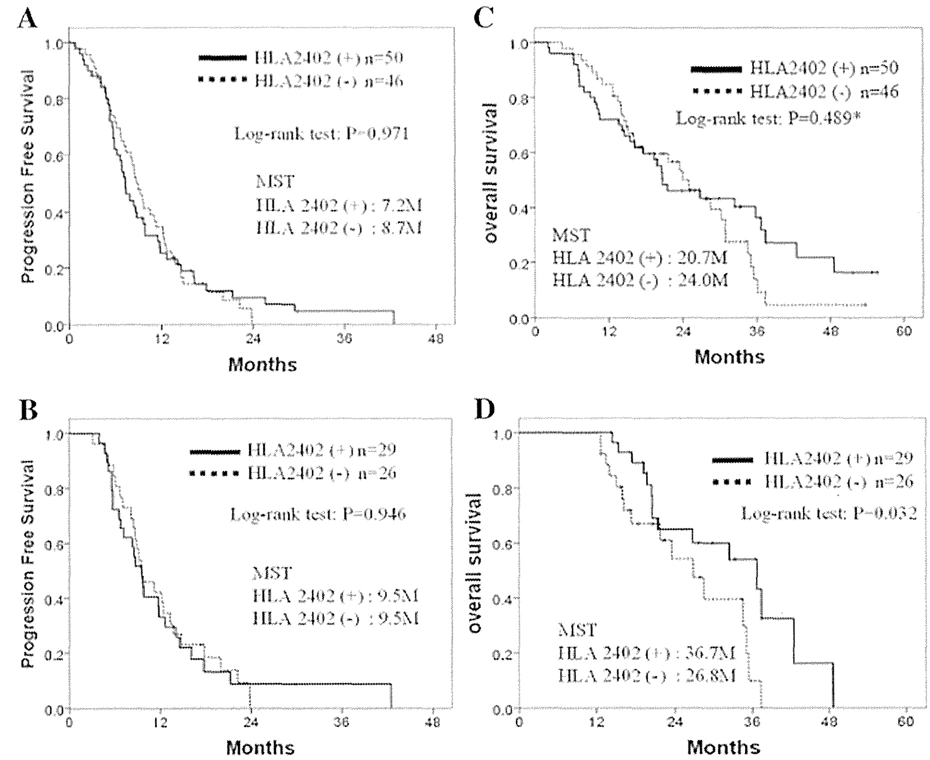


**Figure 2 Progression free survival and overall survival.** **A** and **B**, comparison of progression free survival between HLA-A*2402-mached and -unmatched groups; **A**, all patients; **B**, the patients who received the vaccination for more than 12 months. **C** and **D**, comparison of overall survival between HLA-A*2402-matched and -unmatched groups; **C**, all patients; **D**, patients who received the vaccination for more than 12 months. MST, median survival time; HLA, human leukocyte antigen; M, months; *the weighted log-rank tests with the Harrington-Fleming class of weights were performed and resulted in, $\rho = 0$, and $\gamma = 0.5$, $p = 0.186$; $\rho = 0$, and $\gamma = 1$, $\rho = 0.080$; $\rho = 0$, and $\gamma = 2$, $\rho = 0.101$.

the patients who received the vaccination for more than 12 months, but there was also no difference between these two groups (Figure 2B, P = 0.946).

### Overall survival

The median OS was calculated to be 20.7 months in the HLA-A*2402-matched group and 24.0 months in the unmatched group. There was no significant difference between the two groups (Figure 2C; log-rank test, $p = 0.489$; Harrington-Fleming method, $\rho = 0$ and $\gamma = 0.5$, $p = 0.186$; $\rho = 0$ and $\gamma = 1$, $p = 0.080$; $\rho = 0$ and $\gamma = 2$, $p = 0.101$). Interestingly, when the patients were able to receive the vaccination for more than 12 months, the OS of the HLA-A*2402-matched group was significantly better than that of the unmatched group (Figure 2D; log-rank test, $p = 0.032$).

### Safety

The most common adverse events (AEs) observed in this trial were neurologic toxicity and hematologic toxicities (Table 3). There was no significant difference in the incidence of AEs including injection site reaction in the two groups. Although the incidences of serious adverse events (SAEs) were almost similar in the two groups, that of neutropenia was relatively higher in the HLA-A*2402-matched group than the unmatched group. Interstitial pneumonia that led to the death was observed in two cases in the HLA-matched group and in one case in the HLA-unmatched group (Table 4).

### Immunological biomarkers

NLR is defined as the neutrophil to lymphocyte ratio, and in this study we categorized the patients into two groups (< 3 and ≧ 3) according to the papers reported previously [27]. In this study, NLR of <3.0 was a prognostic marker for the longer survival with peptide cocktail and oxaliplatin-containing chemotherapy (Figure 3A; log-rank test, $p = 0.043$). The Lymphocyte-% of ≧ 15% was also associated with a long survival (Figure 3B; log-rank test, $p = 0.034$). Hence, we examined the combined effect of each of these two markers and the HLA types on the clinical efficacy of the vaccination. In patients with a NLR of < 3.0, a significantly longer overall survival was observed in the HLA-A*2402-matched group than the HLA-A*2402-unmatched group (Figure 3C; log-rank test, $P = 0.289$; Harrington-Fleming method, $\rho = 0$ and $\gamma =$

**Table 3 Frequent and Severe Adverse Events (CTCAE version 3.0)**

| | FOLFOX (n = 89), FOLFOX + Bev (n = 4), XELOX + Bev (n = 1) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HLA-A*2402-matched (n = 50) | | | | | | | | | | HLA-A*2402-unmatched (n = 46) | | | | | | | | | |
| | FOLFOX (n = 48), XELOX (n = 2) | | | | | | | | | | FOLFOX (n = 41) + Bev (n = 4), XELOX + Bev (n = 1) | | | | | | | | | |
| Adverse Event | 1 | | 2 | | 3 | | 4 | | 5 | | 1 | | 2 | | 3 | | 4 | | 5 | |
| | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** | **No** | **%** |
| Hand-foot syndrome | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| Allergy | 4 | 8 | 3 | 6 | 2 | 4 | 0 | 0 | 0 | 0 | 3 | 7 | 4 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| Mucositis | 2 | 4 | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nausea/vomiting | 5 | 10 | 1 | 2 | 2 | 4 | 0 | 0 | 0 | 0 | 6 | 13 | 2 | 4 | 1 | 2 | 0 | 0 | 0 | 0 |
| Neurologic toxicity | 15 | 30 | 10 | 20 | 4 | 8 | 0 | | 0 | 0 | 17 | 37 | 10 | 22 | 5 | 11 | 1 | 2 | 0 | 0 |
| Anorexia | 10 | 20 | 3 | 6 | 4 | 8 | 0 | 0 | 0 | 0 | 10 | 22 | 4 | 9 | 2 | 4 | 0 | 0 | 0 | 0 |
| Diarrhea | 3 | 6 | 6 | 12 | 2 | 4 | 0 | 0 | 0 | 0 | 3 | 7 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| Fatigue/Asthenia | 5 | 10 | 1 | 2 | 2 | 4 | 0 | 0 | 0 | 0 | 5 | 11 | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 |
| Fever | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 7 | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Injection site reaction | 18 | 36 | 18 | 36 | 9 | 18 | 0 | 0 | 0 | 0 | 20 | 43 | 17 | 37 | 3 | 13 | 0 | 0 | 0 | 0 |
| Interstitial pneumonia | 0 | 0 | 0 | 0 | 4 | 8 | 0 | 0 | 2 | 4 | 0 | 0 | 0 | 0 | 4 | 9 | 0 | 0 | 1 | 2 |
| Neutropenia | 5 | 10 | 10 | 20 | 10 | 20 | 1 | 2 | 0 | 0 | 8 | 17 | 14 | 30 | 2 | 4 | 1 | 2 | 0 | 0 |
| Leukopenia | 10 | 20 | 12 | 24 | 1 | 2 | 0 | 0 | 0 | 0 | 12 | 26 | 9 | 20 | 2 | 4 | 0 | 0 | 0 | 0 |
| Thrombocytopenia | 17 | 34 | 3 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 43 | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bilirubin | 2 | 4 | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| AL-P | 11 | 22 | 1 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 10 | 22 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Creatinine | 3 | 6 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Hemoglobin | 11 | 22 | 5 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 28 | 7 | 15 | 0 | 0 | 0 | 0 | 0 | 0 |
| Embolism | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 |
| AST/ALT | 12 | 24 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 6 | 13 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

No gastrointestinal perforation nor bleeding wound healing complication was observed. FOLFOX, infusional fluorouracil, leucovorin, and oxaliplatin; XELOX, capecitabine and oxaliplatin; Bev, bevacizumab; AL-P, alkaline phosphatese; AST, aspartete aminotransfarase; ALT, alanine aminotransferase; CTCAE, the Common Terminology Criteria for Adverse Event version 3.0; HLA, Human leukocyte antigen.

0.5, p = 0.152; ρ = 0 and γ = 1, p = 0.064; ρ = 0 and γ = 2, p = 0.035) while this difference was not observed in patients with NLR of ≧ 3.0 (log-lank test, p = 0.962; Harrington-Fleming method, ρ = 0 and γ = 0.5, p = 0.495; ρ = 0 and γ = 1, p = 0.346; ρ = 0 and γ = 2, p = 0.251). Similarly, in a patient group with a lymphocyte% of > 15%, a longer overall survival was observed in the HLA-A*2402-matched group (Figure 3D; log-lank test, p = 0.340; Harrington-Fleming method, ρ = 0 and γ = 0.5, p = 0.114; ρ = 0 and γ = 1, p = 0.051; ρ = 0 and γ = 2, p = 0.029).

## Discussion

We performed a phase II study using a cocktail of five epitope peptides, which we previously confirmed its safety, together with oxaliplatin-based chemotherapy. The cocktail contained three peptides derived from three oncoantigens and two peptides targeting VEGFR1 and VEGFR2. This study was an HLA-A-status double-blind, phase II study of five therapeutic epitope-peptides with oxaliplatin-based chemotherapy as a first-line therapy for advanced

**Table 4 Interstitial Pneumonia**

| HLA genotype | CTCAE grade | Result of DLLT |
|---|---|---|
| 2402/2402 | 3 | 5FU |
| 2402/1101 | 3 | negative |
| 2402/1101 | 5 | negative |
| 2402/0206 | 3 | negative |
| 2402/2603 | 3 | 5FU |
| 2402/2602 | 5 | negative |
| 1101/2601 | 3 | 5FU |
| 2601/3101 | 3 | 5FU |
| 1101/3101 | 3 | 5FU |
| 3004/3303 | 5 | not examined |
| 1101/3101 | 3 | not examined |

CTCAE, the Common Terminology Criteria for Adverse Event version 3.0; HLA, Human leukocyte antigen; DLTT, drug-induced lymphocyte transformation test; 5FU, 5-fluorouracil.

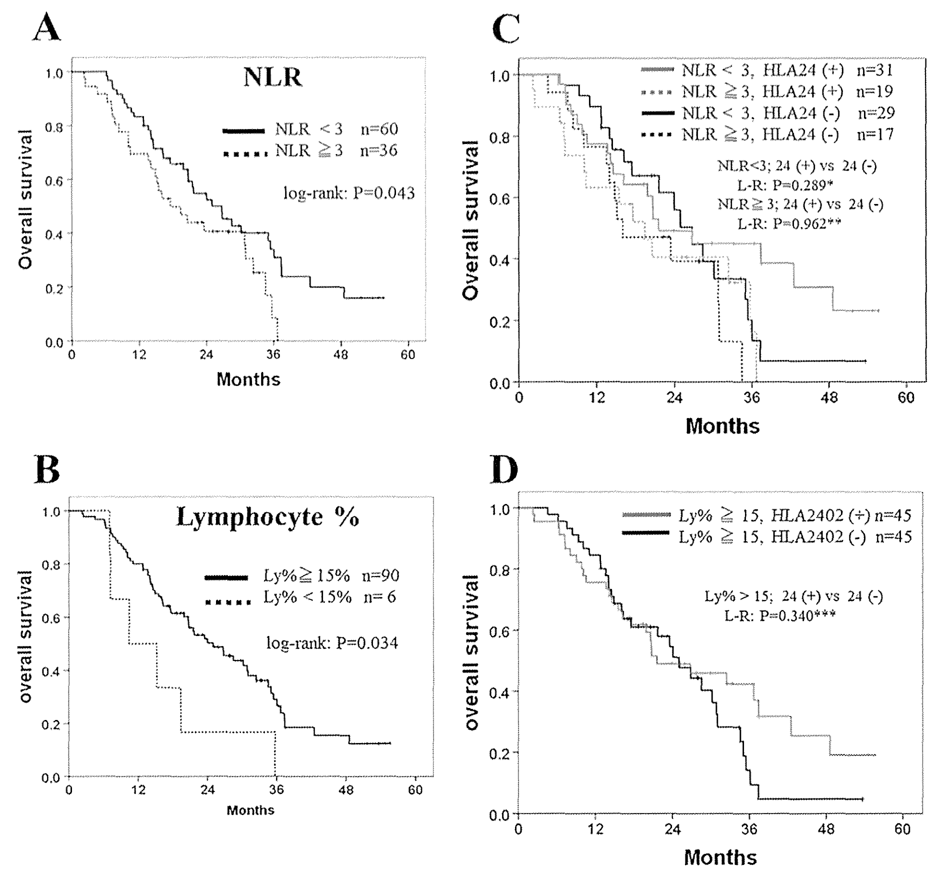


**Figure 3 Biomarkers for the survival and the clinical efficacy of vaccination.** Neutrophil/lymphocyte ratio (NLR) < 3.0 and Lymphocyte-% ≧ 15% were considered as indicative factors. **A** and **B**, comparison between the favorite group and others. **C**, comparison of the patients with a NLR of ≧ 3 or a NLR of <3 between the HLA-A*2402-matched and -unmatched groups. **D**, comparison of the patients with Lymphocyte-% ≧ 15% between the HLA-A*2402 positive and negative groups. Lymphocyte (Ly)-%, the percentage of lymphocytes among the peripheral leukocytes; NLR, neutrophil/lymphocyte ratio; HLA, human leukocyte antigen; L-R, log-rank test; *the weighted log-rank tests with the Harrington-Fleming class of weights were performed and resulted in, $\rho = 0$, and $\gamma = 0.5$, $p = 0.152$; $\rho = 0$, and $\gamma = 1$, $p = 0.064$; $\rho = 0$, and $\gamma = 2$, $p = 0.035$; **the Harrington-Fleming tests were resulted in, $\rho = 0$, and $\gamma = 0.5$, $p = 0.495$; $\rho = 0$, and $\gamma = 1$, $p = 0.346$; $\rho = 0$, and $\gamma = 2$, $p = 0.251$; *** the Harrington-Fleming tests were resulted in, $\rho = 0$, and $\gamma = 0.5$, $p = 0.114$; $\rho = 0$, and $\gamma = 1$, $p = 0.051$; $\rho = 0$, and $\gamma = 2$, $p = 0.029$.

colorectal cancer (FXV study). In this study, we observed many interesting results.

Firstly, the OS of the HLA-A*2402-matched group was significantly higher compared to that of the unmatched group (log-rank test, p = 0.032) when patients who received the vaccination for more than 12 months (Figure 2D) although no difference in PFS was observed between the two groups (Figures 2B). These results indicated that the additional effect of vaccination on the standard chemotherapy was likely to be slow-acting as this kind of delayed response by the vaccine treatment was indicated in the guidance for therapeutic cancer vaccines released from the US Food and Drug Administration in October, 2011 [28].

Secondly, neutrophil/lymphocyte ratio (NLR) might become a prognostic marker for patients who received the peptide vaccine in combination with standard chemotherapy (Figure 3A, log-rank; p = 0.043), and there was an obvious tail effect for extremely long survival. Then we examined the efficacy of vaccination by comparing HLA-matched group and -unmatched group. In patients with an NLR of < 3.0, a significantly longer survival in the HLA-matched group than the HLA-unmatched group was observed (Figure 3B; log-rank, p = 0.289; Harrington-Fleming, p = 0.035), while this difference was not observed in the two groups with NLR of ≧ 3.0 (log-rank, p = 0.962; Harrington-Fleming, p = 0.251). This result also support the idea that it may be critically important to apply vaccine treatment to patients with better immune status, and NLR might be a one of good predictive markers to select the appropriate patient populations for this type of treatment. A similar result was observed when we analyzed patients with lymphocyte% of ≧ 15%; HLA-matched patients with lymphocyte% of ≧ 15 showed significantly better prognosis than HLA-unmatched patients (Figure 3D; log-rank, p = 0.340; Harrington-Fleming, p = 0.029). The selection of patients with lower NLR and higher lymphocyte percentage might be useful to the selection of patients who are likely to respond well to vaccine treatment and improve clinical outcomes.

Vaccinations with a cocktail of five peptides together with oxaliplatin-based chemotherapy in metastatic CRC patients were well tolerated, except for relatively frequent cases (11 cases; 11.4%) of pneumonitis (Tables 3 and 4), whose incidence seemed to be higher than previously reported for oxaliplatin-based chemotherapies although no difference was observed between HLA-matched and -unmatched group. Correale et al. reported two cases (5.5%) in 36 patients with advanced gastric cancer treated with gemcitabine plus oxaliplatin, folinic acid, and 5-fluorouracil (FOLFOX-4) [29]. Usui et al. reported that four cases (3.9%) of pneumonitis among 104 Japanese patients treated with oxaliplatin-containing regimes for advanced colorectal cancer [30]. In addition, there have been many case reports of oxaliplatin-related pneumonitis [31-35]. In this study, eleven (11.4%) of 96 patients suffered from severe pneumonitis including three cases with grade 5 pneumonitis. To investigate the possible cause of pneumonitiswe performed drug-induced lymphocyte transformation test (DLTT) for nine patients whose samples were available. Among them, five patients (55.6%) were judged to be positive to fluorouracil alone, and the remaining four patients were negative for all of the antigens tested. Although the size of this study is not large enough to make any conclusion and there is no difference between the two groups, this adverse event should be carefully monitored when we will perform the next-step clinical trial.

Although the efficacy of our peptide vaccine was not clearly demonstrated in this phase II study, the timing of and combination treatment with vaccination might not be optimized, and the sample size was limited. Recently, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are reported as potent immunosuppressive cells to protect cancer cells from the host immune system [36,37]. Over expression of PD-L1and PD-1 as well as up-regulation of indoleamine-2,3-dioxygenase (IDO) in the tumor microenvironment also inhibit the CTL functions [38]. Hence, to overcome these immune-escape mechanisms, various approaches have been taken in the last decade [39,40]. For example, anti-PD1antibody [41], anti-PD-L1antibody [42], and anti-CTL4 antibody [43] were applied in clinical trials to overcome the suppressive immuno checkpoints, and surprisingly high objective response rates were observed in many types of malignant neoplasm. Small-molecule inhibitors [44] that block IDO enzymatic activity or cyclophosphamide to reduce the number of Tregs [45] were also applied in clinical trials to dissolve the suppressive immunity. For the successful next generation immunotherapy, peptide vaccine should be combined with some agents to modify the immune-suppressive tumor microenvironments.

In conclusion, our cocktail of five therapeutic epitope peptides appears to be effective in a subset of patients, and warrants a randomized phase III study. In the phase III study, biomarkers such as NLR and lymphocyte-% might be useful for assessing the response to the peptide vaccine and for selecting patients likely to have a better treatment outcome with the vaccination.

## Conclusions

This phase II cancer vaccine therapy demonstrated that our therapeutic peptides cocktail was likely to be effective in a subset of patients and warrants a randomized phase III study. In the phase III study, predictive biomarkers such as NLR and lymphocyte-% should be used for its response and for selecting patients to have a better treatment outcome with the vaccination.

## Additional file

**Additional file 1: Summary of the protocol.**

#### Abbreviations

RNF43: Ring finger protein 43; TOMM34: 34 kDa-translocase of the outer mitochondrial membrane; KOC1: insulin-like growth factor–II mRNA binding protein 3; VEGFR: Vascular endothelial growth factor receptor; HPLC: High performance liquid chromatography; CRC: Colorectal cancer; ELISPOT: Enzyme-linked immunospot; PBMC: Peripheral blood mononuclear cells; CTL: Cytotoxic T lymphocytes; RR: Response rates; CR: Complete clinical response; SD: Stable disease; PD: Progressive disease; PFS: Progression free survival; OS: Overall survival; HLA: Human leukocyte antigen; MST: Median overall survival time; ECOG: Eastern cooperative oncology group; RECIST: Response evaluation criteria in solid tumors; TIL: Tumor infiltrating cells; CTCAE: Common terminology criteria for adverse events version3.0; AEs: Adverse events; SAEs: Serious adverse events; DLTT: Drug-induced lymphocyte transformation test; PS: Performance status; IFA: Incomplete freund's adjuvant; CT: Computed tomography; MRI: Magnetic resonance imaging; NLR: Neutrophil/lymphocyte ratio; FOLFOX: Infusional fluorouracil, leucovorin, and oxaliplatin; XELOX: Capecitabine and oxaliplatin, Tregs, regulatory T cells; MDSCs: Myeloid-derived suppressor cells; IDO: Indoleamine-2,3-dioxygenase.

#### Competing interests

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.

#### Authors' contributions

SH designed, performed and evaluated clinical study, and wrote the manuscript. YN and MO participated in the design, review and revision of the manuscript. HT, KH, KT, RS, HO, RE, FS, KO, TF, TN, KS, KY, YI, SK, YS, NS, SY, HS, AK, TF, YK and HF assisted to perform clinical study. RT, HT, and TY contributed in the data collection and statistical analysis. All authors participated in the data acquisition and discussion of the manuscript and approved the final manuscript.

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**Author details**
[1]Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Ube, Japan. [2]Department of Medicine and Surgery, The University of Chicago, Chicago, IL, USA. [3]Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan. [4]Department of Surgery, Kure-Kyosai Hospital, Kure, Japan. [5]Department of Surgery, Ogori Daiichi General Hospital, Yamaguchi, Japan. [6]Department of Surgery, Kinki University Faculty of Medicine, Osaka-Sayama, Japan. [7]Department of Surgery, Kanmon-Medical Center, Shimonoseki, Japan. [8]Department of Surgery, Shimonoseki-Kosei Hospital, Shimonoseki, Japan. [9]Tokyo Women's Medical University Medical Center East, Tokyo, Japan. [10]Department of Pharmacy, Yamaguchi University Hospital, Ube, Japan. [11]Department of Biostatistics, National Cancer Center, Chiba, Japan. [12]Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan.

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