Phase II Study of Personalized Peptide Vaccination for Previously Treated Advanced Colorectal Cancer

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Running title: Personalized Peptide Vaccine in Advanced Colorectal Cancer

Key words: colorectal cancer, peptide vaccine, biomarker, multivariate Cox regression analysis, genetic polymorphism

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Word count: 4982

Total numbers of figures and tables: 7

Supplementary material: 8 (Methods, 1; Figure, 2; Table, 5)

Abstract

Prognosis of advanced colorectal cancer (aCRC) remains poor and development of new therapeutic approaches, including immunotherapy, is needed urgently. In the current study, we conducted a phase II study of personalized peptide vaccination (PPV) in 60 previously treated aCRC patients, who had failed at least one regimen of standard chemotherapy and/or targeted therapy. For PPV, a maximum of four HLA-matched peptides were individually selected from a pool of 31 different peptide candidates based on the pre-existing host immunity, and administered subcutaneously without severe adverse events. Boosting of IgG and cytotoxic T lymphocyte (CTL) responses specific to the administered peptides was observed in 49% and 63%, respectively, of the patients, who completed the first cycles of six vaccinations. Median overall survival (OS) time was 498 days with one- and two-year survival rates of 53% and 22%, respectively. Multivariate Cox regression analysis of pre-vaccination factors showed that plasma IL6, IP-10, and BAFF levels were significantly prognostic for OS [hazard ratio (HR) =1.508, P = 0.043; HR = 1.579, P = 0.024; HR = 0.509, P = 0.002; respectively]. In addition, increased peptide-specific CTL responses after vaccination were significantly predictive of favorable OS (HR = 0.231, P = 0.021), suggesting a causal relationship between biological and clinical efficacy of PPV. Based on the safety profile and potential clinical efficacy, we believe clinical trials of PPV would be warranted for previously treated aCRC patients.

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer death in the world. Although recent advances in chemotherapy and/or targeted therapy have helped to improve the clinical outcomes of patients with advanced CRC (aCRC), the prognosis still remains poor (1). Therefore, development of new therapeutic approaches, including immunotherapy, would be highly desirable. However, limited numbers of clinical trials of immunotherapies have been reported for aCRC patients (2,3).

We have developed a novel approach of cancer immunotherapy, named personalized peptide vaccination (PPV), in which vaccine peptides were selected from 31 cytotoxic T lymphocyte (CTL) epitope peptides derived from 15 tumor-associated antigens (TAA), based on both HLA-class IA-types and pre-existing host immunity (4,5). Recently conducted clinical trials of PPV for patients with various types of cancers demonstrated the feasibility of this new approach (4-7). For aCRC patients, phase I studies showed the safety and immunogenicity of PPV combined with chemotherapeutic agents, along with possible prolongation of survival time in immunologic responders (8,9). In the present study, we conducted a phase II study to examine the feasibility of PPV and to identify biomarkers that would be useful for prediction of overall survival (OS) in previously treated aCRC patients.

Materials and Methods

Patients

Previously treated aCRC patients, who had failed at least one regimen of standard chemotherapy and/or targeted therapy, were eligible for inclusion in the present study, if they had positive humoral responses as determined by the peptide-specific IgG titers to at least two of the 31 different candidate vaccine peptides (Supplementary Table S1) (4-9). Other inclusion and exclusion criteria are shown in Supplementary Methods. The protocol was approved by the Kurume University Ethics Committee, and was registered in the UMIN Clinical Trials Registry (UMIN000006493). After a full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol

This was an open-label phase II study in which the endpoints were to analyze the clinical feasibility and safety of PPV and to identify biomarkers useful for prediction of OS after PPV in aCRC patients. Thirty-one vaccine peptide candidates, whose safety and immunologic effects had been confirmed in clinical studies conducted previously (4-9), were prepared under the conditions of Good Manufacturing Practice (GMP) by the PolyPeptide Laboratories (San Diego, CA) and American Peptide Company (Vista, CA). Expressions of vaccine antigens in CRC tissues were examined by immunohistochemistry (Supplementary Fig. S1).

Of the 15 vaccine antigens employed for PPV, 13 were detectable in CRC tissues tested, but not the two prostate-related antigens (PSA and PSMA) (Supplementary Table S1).

The protocol consisted of two cycles of six vaccinations. Two to four HLA-matched peptides were selected from the 31 peptides in individual patients, based on pre-existing host immunity before vaccination by assessing the titers of IgG specific to each peptide, as described previously (4-9). The peptides derived from PSA and PSMA were selected only when pre-existing IgG responses to other remaining peptides were absent. The selected peptides (3 mg/each peptide) were administered subcutaneously with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for six consecutive weeks. After the completion of the first cycle of six vaccinations, IgG titers specific to each of 31 peptide candidates in plasma from vaccinated patients were measured again, and two to four HLA-matched peptides with higher specific IgG titers were selected and administered six times every two weeks for the second vaccination cycle. After the second cycle, vaccinations were maintained, if the patients wished; two to four antigen peptides, which were re-selected based on the titers of peptide-specific IgG at every cycle of six vaccinations, were administered every four weeks until uncontrollable disease progression. Combined chemotherapies and/or targeted therapies were allowed during the vaccination period. Adverse events (AE) were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.0. Complete blood counts

and serum biochemistry tests were performed before and after every six vaccinations.

Measurement of humoral and cellular immune responses

Peripheral blood (30 ml) was obtained from the vaccinated patients before and after each cycle of six vaccinations. After centrifugation, plasma was separated and stored frozen until analysis. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) and stored frozen until analysis. Post-vaccination blood samples were available from 51 and 35 patients at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively.

Humoral immune responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay with the Luminex 200 system (Luminex, Austin, TX), as reported previously (10,11). CTL responses specific to the vaccine peptides were evaluated by interferon-γ (IFNγ) ELISPOT assay. The detailed procedures are shown in the Supplementary Methods. When spot-numbers in response to specific peptides were significantly higher ($P < 0.05$ by Student's t-test) than those in response to the control peptides, antigen-specific CTL responses were shown as the differences between them (means of the triplicate samples).

Measurement of laboratory markers

Levels of C-reactive protein (CRP), serum-amyloid A (SAA), and IL6 in pre-vaccination plasma were examined by ELISA using kits from R&D systems (Minneapolis, MN), Life Technologies (Carlsbad, CA), and eBioscience (San Diego, CA), respectively. Bead-based multiplex assays were used to measure cytokines, including IL4, IL13, IL21, IP-10 (IFNγ-induced protein 10), BAFF (B-cell activating factor), and TGF-β, with the Luminex 200 system. Pre-vaccination plasma from one patient was unavailable for this analysis (n = 59). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. Means of the duplicate samples were used for statistical analysis.

IL6, IL6 receptor (IL6R), and CRP genetic polymorphisms

DNA was extracted from thawed PBMCs using a QIAamp Blood kit (Qiagen, Hilden, Germany) and stored at –80°C until analysis. To investigate the IL6 -634G>C (rs1800796), CRP 1846C>T (rs1205), and IL6R 48892A>C (rs8192284, Asp358Ala) genetic polymorphisms with the extracted DNA, genotyping was performed using the polymerase chain reaction-restriction fragment length polymorphism method, as reported previously (12,13).

Statistical analysis

OS time was defined as duration from the first date of peptide vaccination or that of the first-line chemotherapy until the date of death and was censored by the last date of contact for patients alive at the last follow-up. The survival function, including survival rates, for OS was estimated by the Kaplan-Meier method with the Greenwood variance estimates. In addition, exploratory analyses, which were not pre-defined in the protocol, were performed to examine association among biomarkers, immune responses, and OS. Association between pre-vaccination biomarkers and OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. In applying Cox regression, the transformation of log(biomarker+1) was employed since the distribution of each biomarker was highly skewed. Statistically significant biomarkers ($P < 0.1$) in the univariate analysis were included in the multivariate analysis. Spearman's rank correlation among these biomarkers was estimated to avoid collinearity.

Humoral and cellular immune responses were determined by IgG and CTL responses specific to the administered peptides, respectively. IgG responses were defined as positive if IgG titers specific to at least one of the administered peptides in the post-vaccination plasma were more than 2-times higher than those in the pre-vaccination plasma, and as negative otherwise. CTL responses were defined as positive if CTL responses to at least one of the administered peptides in the post-vaccination PBMCs were greater than those in the pre-vaccination PBMCs and as negative otherwise. Association between IgG or CTL

responses and other prognostic factors was examined by logistic regression analysis. Association between IgG or CTL responses and OS was examined by Kaplan-Meier method with the log-rank test and the Cox regression analysis. The relationship between IgG and CTL responses was evaluated by Chi-square test. The prognostic significance of genetic polymorphisms was analyzed by Kaplan-Meier survival curves with log-rank test. All statistical tests were conducted at two-sided 5% significance level unless indicated. Due to the exploratory nature of biomarker analyses, any multiplicity adjustment was not applied. All statistical analyses were conducted using the JMP version 10 or SAS version 9.3 software package (SAS Institute Inc., Cary, NC).

Results

Patient characteristics

Between January 2009 and November 2012, 60 patients with aCRC were enrolled in this study. Table 1 summarizes the clinicopathologic characteristics of the enrolled patients. There were 33 male and 27 female subjects with a median age of 60 years, ranging from 35 to 83 years. All patients (stage IV, n = 26; recurrent, n = 34) were refractory to at least one regimen of chemotherapies and/or targeted therapies. The location of original tumor was right-sided colon (n = 14) or left-sided colon/rectum (n = 46). All patients had metastatic tumors; liver (n = 33), lung (n = 31), peritoneal dissemination (n = 23), or lymph nodes (n=14). The number of metastatic organs per patient was one (n = 29), two (n = 21), or three (n = 10). Before enrollment, the patients had failed to respond to one (n = 17), two (n = 15), three (n = 9), four (n = 13) or five (n = 6) regimen(s) of chemotherapies, targeted therapies, and/or combinations of them. The median duration of these preceding regimens prior to PPV was 552.5 days, ranging from 9 to 1819 days. The median time from patient enrolment to first vaccination was 13.5 days, ranging from 7 to 27 days. The numbers of peptides used for vaccination during the first cycle were four peptides in 36 patients, three in 16 patients, and two in 8 patients. Among the 60 patients, 51 (85%) completed the first cycle of six vaccinations, and the remaining 9 patients failed to do so due to rapid disease progression. The median number of vaccinations was 12, with a range of 2 to 33. During the PPV, 49 patients (82%) received

combined chemotherapies and/or targeted therapies, including FOLFOX/XELOX with bevacizumab (n = 10), FOLFIRI with bevacizumab (n = 5), FOLFIRI (n = 5), S-1 (n = 5), irinotecan with cetuximab (n = 5), cetuximab (n = 5), FOLFOX/XELOX (n = 2), FOLFIRI with cetuximab (n = 2), or other regimens (n = 10). The remaining 11 patients (18%) had no options for combined chemotherapies or were unable to tolerate them.

Adverse events

Toxicities are shown in Supplementary Table S2. The most frequent AEs were dermatologic reactions at the injection sites (n = 55, 92%), anemia (n = 27, 45%), lymphopenia (n = 23, 38%) and hypoalbuminemia (n = 20, 33%). Grade 4 anemia was noted in two patients. Grade 3 serious adverse events (SAEs) comprised leukocytopenia (n = 3), lymphopenia (n = 2), increased γ-glutamyl transpeptidase (n = 2), hyponatremia (n = 2), ileus (n = 2), increased AST (n = 1), hyperglycemia (n = 1), hypercholesteremia (n = 1), and rash (n = 1). However, according to the evaluation by the independent safety evaluation committee for this trial, all the grade 3 or 4 SAEs were concluded to be not directly associated with the vaccinations, but with other causes, such as combined chemotherapies and/or targeted therapies and cancer progression.

Clinical outcomes

Median OS time (MST) for the 60 patients from the first vaccination was 498 days (95% confidence interval, 233 - 654 days) with one- and two-year survival rates of 53% and 22%, respectively (Fig. 1A). When calculated from the first date of the first-line chemotherapy, MST was 1179 days (95% confidence interval, 885 - 1272 days) with one-, two-, three-, four-, and five-year survival rates of 97%, 77%, 53%, 24%, and 15%, respectively (data not shown). Of note, among the enrolled 60 patients, 32 patients, who had a treatment history of two or more regimens of standard chemotherapy and were refractory or intolerant to all of irinotecan, oxaliplatin, and fluoropyrimidines before enrollment, showed MST of 375 days (95% confidence interval, 191 - 561 days) from the first vaccination, with one-year survival rate of 51% (Fig. 1B).

Relationship between pre-vaccination clinical findings or laboratory data and OS

The Cox proportional hazards model was used to identify factors that were significantly associated with OS, from pre-vaccination clinical findings or laboratory data. As shown in Table 2, univariate analysis using pre-vaccination clinical findings showed that the number of previous chemotherapy regimens were potentially prognostic factors ($P = 0.067$). In addition, albumin, CEA, CRP, SAA, IL6, IP-10, and BAFF in pre-vaccination blood were significantly prognostic of OS by univariate analysis ($P = 0.012$, $P = 0.002$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.018$, and $P = 0.005$, respectively). However, none of the other factors examined were

significantly correlated with OS.

Multivariate Cox regression analysis was performed to evaluate the influence of each of the factors that had been shown to be significantly associated with OS in the univariate analysis ($P < 0.1$). SAA and CRP were not included in this analysis, since the level of SAA and CRP was highly correlated with that of IL6 (SAA vs IL6: Spearman rank correlation coefficient = 0.482; CRP vs IL6: Spearman rank correlation coefficient = 0.653). As shown in Table 2, higher IL6 and IP-10 levels and a lower BAFF level in pre-vaccination plasma was significantly predictive of unfavorable OS [hazard ratio (HR) for the unit of 1 SD = 1.508, 95% confidence interval (CI) = 1.014 - 2.245, P = 0.043; HR = 1.579, 95% CI = 1.062 - 2.347, P = 0.024; HR = 0.509, 95% CI = 0.329 - 0.787, P = 0.002; respectively]. The other factors showed no statistically significant association.

Relationship between IL6, IL6R, or CRP genetic polymorphisms and OS

Since inflammation markers, IL6 and CRP, were potentially prognostic in patients treated with PPV, we examined genetic polymorphisms of related genes, IL6 -634G>C, CRP 1846C>T, and IL6R 48892A>C (Supplementary Table S3). There was no statistically significant relationship between IL6 634G>C polymorphism and OS (P = 0.319). However, CRP 1846C>T and IL6R 48892A>C polymorphisms tended to show a statistically significant effect on OS (P = 0.069 and P = 0.085, respectively). Patients carrying the CRP 1846C/C

genotype had a potentially better prognosis than those carrying the CRP 1846C/T or those carrying the CRP 1846T/T genotype (P = 0.029 or P = 0.054, respectively) (Fig. 2A). In addition, patients carrying the IL6R 48892C/C or 48892A/C genotypes tended to show a better prognosis than those carrying the IL-6R 48892A/A genotype (P = 0.059) (Fig. 2B). This genetic polymorphism was further evaluated in patients positive or negative for IL6 in pre-vaccination plasma (Fig. 2C). Of note, the difference between patients carrying the IL-6R 48892C/C or A/C genotypes and the IL-6R 48892A/A genotype was statistically significant in patients negative for plasma IL6 (P = 0.025), but not in those positive for plasma IL6 (P = 0.118).

Immune responses to the vaccine peptides

IgG responses specific to at least one of the administered peptides were increased in 25 of 51 patients (49%) and in 33 of 35 patients (94%) at the end of the first and second cycles of vaccinations, respectively (Supplementary Table S4). CTL responses specific to at least one of the administered peptides that were evaluated by IFNγ ELISPOT assay were increased in 32 of 51 patients (63%) at the end of the first cycle of vaccinations (Supplementary Table S4). A representative result of IFNγ ELISPOT assay with PBMCs before and after vaccination is shown in Fig. 3A. According to Chi-square test, increased CTL responses against administered peptides after the first cycle of vaccinations were significantly

associated with increased IgG responses (P = 0.002).

Relationship between the increase in peptide-specific CTL or IgG responses after vaccination and other potential prognostic factors, including pre-vaccination IL6, IP-10 and BAFF levels (Table 2), were examined by logistic regression analysis. As shown in Table 3, the level of IP-10 was predictive of the increase in CTL and IgG responses (odds ratio, 0.427; 95% CI, 0.191 - 0.957; P = 0.039; odds ratio, 0.354; 95% CI, 0.127 - 0.982; P = 0.046; respectively), whereas other factors, including IL6 and BAFF levels, were not predictive.

Prognostic significance of boosting of peptide-specific CTL and IgG responses

The prognostic significance of successful boosting of peptide-specific CTL or IgG responses was analyzed by Kaplan-Meier survival curves with log-rank test. This analysis showed a statistically significant association between increased CTL or IgG responses and OS (P = 0.025 and P = 0.022, respectively) (Fig. 3B and 3C). Patients with both CTL and IgG responses (P = 0.010), but not those with CTL responses alone (P = 0.138) or IgG responses alone (P = 0.351), showed significantly better prognosis than those without CTL or IgG responses (Supplementary Fig. S2).

In addition, multivariate Cox regression analysis with peptide-specific CTL or IgG responses (positive or negative) and other potential prognostic factors (Table 2) was performed. IP-10 was not included in this analysis because the CTL and IgG responses were

significantly associated with plasma IP-10 level (Table 3). As shown in Table 4, increased CTL responses after vaccination were significantly associated with favorable OS (HR = 0.231, 95% CI = 0.067 - 0.803, P = 0.021) independently of other factors, whereas IgG responses after vaccination were not significantly predictive of favorable OS (HR = 0.790, 95% CI = 0.285 – 2.188, P = 0.650). Furthermore, to analyze association of the magnitude of CTL responses with OS, the number of peptides, to which CTL responses were increased after vaccination, was evaluated by multivariate analysis. As shown in Supplementary Table S5, the number of peptides with increased CTL responses after vaccination was also significantly predictive of favorable OS (HR = 0.216, 95% CI = 0.077 - 0.604, P = 0.004).

Discussion

In the current study, we demonstrated that successful boosting of peptide-specific CTL responses resulted in increased OS after PPV, suggesting a potential clinical benefit of PPV. The most unique aspect of PPV is the personalized selection of optimal antigen peptides for individual patients on the basis of pre-existing host immunity before vaccination (4,5). In view of the heterogeneity of tumors and the complexity and diversity of immune responses, we thought that this approach would be more rational than selecting non-personalized universal tumor antigens. Since tumor tissues were unavailable in most advanced CRC patients, it was difficult to precisely characterize tumor cells in individual patients. Therefore, we selected and administered multiple (up to four) antigens to increase the possibility that the antigens used for vaccination were expressed in tumor cells.

We currently measure pre-existing antigen-specific IgG responses, but not T-cell responses, for personalized selection of antigen peptides from a panel of candidate antigens, because antigen-specific T-cell assays often show limited sensitivity due to quite low frequencies of antigen-specific T cells before vaccinations, even after *in vitro* cell culture for expansion. Indeed, if the pre-existing CTL responses in pre-vaccination PBMCs were used for selection of peptides in the current study, much smaller numbers of peptides would be selected for vaccination (Supplementary Table S4). In contrast, the multiplex bead–based LUMINEX technology allows high-throughput screening of IgG responses specific to large

numbers of peptide antigens with high accuracy (10,11). Our previous studies suggested the clinical significance of antigen-specific IgG responses as a surrogate biomarker in monitoring vaccine-induced immune responses (14). In addition, the current study demonstrated that increased IgG responses against administered peptides after vaccination were significantly associated with increased CTL responses. These results support our hypothesis that evaluation of IgG responses might be useful for predicting peptides that could induce specific CTL responses.

Since the vaccine peptides used for PPV are HLA-restricted CTL epitopes, they might act mainly through peptide-specific CTL responses. Indeed, peptide-specific CTL responses were significantly associated with OS (Table 4). Nevertheless, IgG responses to the vaccine peptides might also affect antitumor immunity. For example, in our preliminary study in mice, antibody-complex with specific peptides facilitated the uptake of peptides and enhanced the cross-presentation of these peptides by antigen-presenting cells (Matsueda S, et al. unpublished data). Further studies are currently in progress for clarification of the biologic functions of peptide-specific IgG.

Since not all patients show clinical benefits from cancer immunotherapies, it would be critical to identify prognostic or predictive biomarkers for patients receiving such therapies. Several post-vaccination biomarkers have been reported to be associated with clinical responses (14-18), but there are currently no validated pre-vaccination predictive biomarkers.

By multivariate analysis, higher IL6 and IP-10 and lower BAFF levels in pre-vaccination plasma were significantly associated with unfavorable OS, although these factors might be prognostic irrespective of treatment, and not necessarily predictive and unique to PPV. Of note, however, the IP-10 level was predictive of the increase in CTL responses, which was associated with improved OS, suggesting that IP-10 might be potentially useful for selecting aCRC patients, who would benefit from PPV. To more clearly assess the causal relation of IP-10, CTL responses, and OS, and to elucidate prognostic vs. predictive relevance of such biomarkers, future randomized, controlled clinical trials with or without PPV would be essential.

IL6 has been reported to induce suppressive immune cell subsets, such as myeloid-derived suppressor cells and Th17 cells (19-22). Therefore, high levels of IL6 might inhibit immune responses to cancer vaccines by inducing these suppressive cells. BAFF is a cytokine for the differentiation and survival of follicular B cells along with humoral response potentiation (23). As previously suggested (24-26), BAFF might induce beneficial humoral immune responses to vaccine antigens. IP-10 is a chemokine for attraction of human monocytes, activated T cells, and NK cells (27,28). Although local production of IP-10 within tumor tissues has been reported to be associated with antitumor immunity, systemic inflammatory responses mediated by IP-10 might contribute to poorer immune responses to

vaccines (27,28). The precise mechanisms of IL6, BAFF, and IP-10 in immune responses after PPV remain to be determined.

Results from the current study suggested that the CRP 1846C>T and IL-6R 48892A>C polymorphisms might show a statistically significant effect on OS after PPV. Since the CRP 1846C>T polymorphism, which affects serum CRP levels (29), has been reported to be associated with advanced diseases in patients with CRC (30) and esophageal squamous cell carcinoma (13), it might be a prognostic factor irrespective of the therapeutic approach. In contrast, since the IL6R 48892A>C polymorphism has been reported to show no effects on prognosis in other types of cancers, such as esophageal squamous cell carcinoma and neuroblastoma, without cancer vaccines (12,31), the prognostic significance of this polymorphism might be unique to PPV vaccinated patients. The IL6R 48892C (358Ala) allele has been reported to affect proteolytic cleavage of the membrane-bound IL6R, leading to reduced numbers of the functioning IL6R (32). As a result, this genetic variant is suggested to contribute to anti-inflammatory effect through attenuation of IL6 signaling on cells expressing the membrane-bound IL6R (33-35). Based on our finding, the effect of reduced IL6R expression might be more prominent when the availability of IL6 is limited, whereas it might be overcome by overexpression of IL6.

Importantly, the current study demonstrated that successful boosting of peptide-specific CTL responses was significantly predictive of favorable OS by multivariate analysis,

suggesting a causal relationship between biological and clinical efficacy of PPV. However, peptide-specific IgG responses were not significantly predictive of OS by multivariate analysis, although they were significantly associated with favorable OS by Kaplan-Meier method with the log-rank test. This discrepancy might be explained by the speculation that IgG responses might be more strongly affected by other confounding factors, such as IL6 and BAFF, compared to CTL responses. Since IL6 and BAFF are known to play important roles in the differentiation and survival of B cells along with humoral response potentiation (19,23), it is possible that they substantially affected IgG responses, but not CTL responses, after vaccination.

In summary, the current study demonstrated that PPV induced substantial immune responses to vaccine antigens without severe adverse events and showed potential clinical benefits in previously treated aCRC patients, even in the refractory stage. Nevertheless, this study has several drawbacks. First, this is a small study with a limited number of patients, all of whom received PPV. Second, combined chemotherapies and/or targeted therapies during the vaccination period might affect the occurrence of immune responses and conclusion about the prognostic versus the predictive role of biomarkers. Therefore, clinical efficacy of PPV, as well as clinical utility of the identified biomarkers, in aCRC patients remain to be confirmed in future larger-scale, randomized trials of PPV without combined chemotherapies or targeted therapies.

Disclosure of Potential Conflicts of Interest

Takanobu Nomura is an employee of the Kyowa Hakko Kirin Co., Ltd. Akira Yamada is a Board member of the Green Peptide Co., Ltd. Kyogo Itoh and Akira Yamada have stock of the Green Peptide Co., Ltd. Kyogo Itoh received research fund from Taiho Pharmaceutical Co., Ltd. No potential conflicts of interests were declared by the other authors.

Acknowledgements

The authors thank Ms. Emi Muta and Ms. Chieko Seki (Kurume University) for their technical assistance.

Grant Support

This study was supported by a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct), Ministry of Education, Culture, Sports, Science and Technology of Japan, and a research program of the Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Table 1. Patient characteristics

Factor		Number
Age (years)		
	Median (range)	60 (35 - 83)
Gender		
	Male	33
	Female	27
Stage		
	Stage IV	26
	Recurrent	34
Location of original tumors		
	Right-sided colon	14
	Left-sided colon or rectum	46
Location of metastatic tumors		
	Liver	33
	Lung	31
	Peritoneal dissemination	23
	Lymph nodes	14
Number of metastatic organs		
	1	29
	2	21
	3	10
Number of previous regimens		
	1	17
	2	15
	3	9
	4	13
	5	6
Duration of previous treatments (days)		
	Median (range)	552.5 (9 - 1819)
HLA type		
	A2	19
	A3	3
	A11	16
	A24	41
	A26	10
	A31	4
	A33	11
Time from patient enrolment till first vaccination		
	Median (range)	13.5 (7 – 27)
Number of vaccinations		
	Median (range)	12 (2 - 33)
Overall survival time (days)		
	Median (95% confidence interval)	498 (223 – 654)

Table 2. Univariate and multivariate analysis for OS with pre-vaccination clinical findings or laboratory data

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age	1.000 (0.972 – 1.029)	0.991		
Gender (male vs female)	1.626 (0.856 – 3.090)	0.138		
Stage (Stage IV vs recurrent)	1.173 (0.622 – 2.212)	0.623		
Number of previous chemotherapy regimens	1.249 (0.985 – 1.584)	0.067	1.279 (0.927 – 1.764)	0.134
Lymphocyte frequency (%)	0.855 (0.661 – 1.172)	0.238		
Hemoglobin (g/dl)	0.834 (0.628 – 1.108)	0.211		
Albumin (g/dl)	0.677 (0.501 – 0.916)	0.012	0.805 (0.451 – 1.437)	0.462
Creatinine (mg/dl)	1.075 (0.779 – 1.485)	0.659		
CEA (ng/dl)	1.754 (1.240 – 2.483)	0.002	1.429 (0.938 – 2.177)	0.096
CRP (ng/ml)	2.525 (1.590 – 4.011)	< 0.001		
SAA (ng/ml)	2.089 (1.433 – 3.046)	< 0.001		
IL-4 (pg/ml)	0.928 (0.667 – 1.292)	0.660		
IL-6 (pg/ml)	1.890 (1.380 – 2.588)	< 0.001	1.508 (1.014 – 2.245)	0.043

IL-13 (pg/ml)	0.963 (0.660 – 1.405)	0.846		
IL-21 (pg/ml)	1.206 (0.909 – 1.600)	0.193		
IP-10 (pg/ml)	1.518 (1.075 – 2.142)	0.018	1.579 (1.062 – 2.347)	0.024
BAFF (pg/ml)	0.599 (0.421 – 0.853)	0.005	0.509 (0.329 – 0.787)	0.002
TGF-β (pg/ml)	1.222 (0.861 – 1.736)	0.261		

Abbreviations: OS, overall survival; CI, confidence interval; CEA, carcinoembryonic antigen; CRP, C-reactive protein; SAA, serum amyloid A; IP-10, interferon gamma-induced protein 10; BAFF, B-cell activating factor

Table 3. Multivariate logistic regression analysis for predicting peptide-specific CTL or IgG responses after vaccination

Factor	CTL responses		IgG responses	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Number of previous chemotherapy regimens	0.996 (0.568 - 1.746)	0.989	1.012 (0.541 - 1.895)	0.970
Albumin (g/dl)	0.640 (0.186 - 2.202)	0.479	2.847 (0.792 - 10.24)	0.109
CEA (ng/dl)	0.772 (0.364 - 1.638)	0.501	1.008 (0.456 - 2.225)	0.985
IL-6 (pg/ml)	0.565 (0.249 - 1.281)	0.172	0.685 (0.281 - 1.668)	0.404
IP-10 (pg/ml)	0.427 (0.191 - 0.957)	0.039	0.354 (0.127 - 0.982)	0.046
BAFF (pg/ml)	0.885 (0.371 - 2.112)	0.783	1.205 (0.492 – 2.954)	0.683

Abbreviations: CTL, cytotoxic T lymphocyte; CI, confidence interval; CEA, carcinoembryonic antigen; IP-10, interferon gamma-induced protein 10; BAFF, B-cell activating factor

Table 4. Multivariate Cox regression analysis for OS

Factor	CTL responses		IgG responses	
	Hazard ratio (95% CI)	***P* value**	**Hazard ratio (95% CI)**	***P* value**
CTL responses (positive vs negative)	0.231 (0.067-0.803)	0.021	NA	NA
IgG responses (positive vs negative)	NA	NA	0.790 (0.285 - 2.188)	0.650
Number of previous chemotherapy regimens	1.171 (0.777 - 1.765)	0.451	1.185 (0.776 - 1.808)	0.432
Albumin (g/dl)	0.577 (0.297 - 1.124)	0.106	0.916 (0.510 - 1.645)	0.769
CEA (ng/dl)	1.884 (1.115 - 3.183)	0.018	2.066 (1.204 - 3.544)	0.008
IL-6 (pg/ml)	1.850 (1.101 - 3.107)	0.020	2.046 (1.220 - 3.432)	0.007
BAFF (pg/ml)	0.400 (0.211 - 0.758)	0.005	0.578 (0.341 - 0.982)	0.043

Abbreviations: OS, overall survival; CTL, cytotoxic T lymphocyte; CI, confidence interval; NA, not assessed; CEA, carcinoembryonic antigen; BAFF, B-cell activating factor

Figure legend

Figure 1. Kaplan-Meier survival analysis.

Curves for overall survival (OS) (solid line) after PPV treatment were estimated by the Kaplan-Meier method in all 60 enrolled patients (A) and in 32 heavily treated patients who were refractory or intolerant to all of irinotecan, oxaliplatin, and fluoropyrimidines before enrollment (B). Dotted lines show 95% confidence intervals. Censored patients are shown as vertical bars.

Figure 2. Prognostic significance of CRP 1846C>T and IL-6R 48892A>C polymorphisms in aCRC patients treated with PPV.

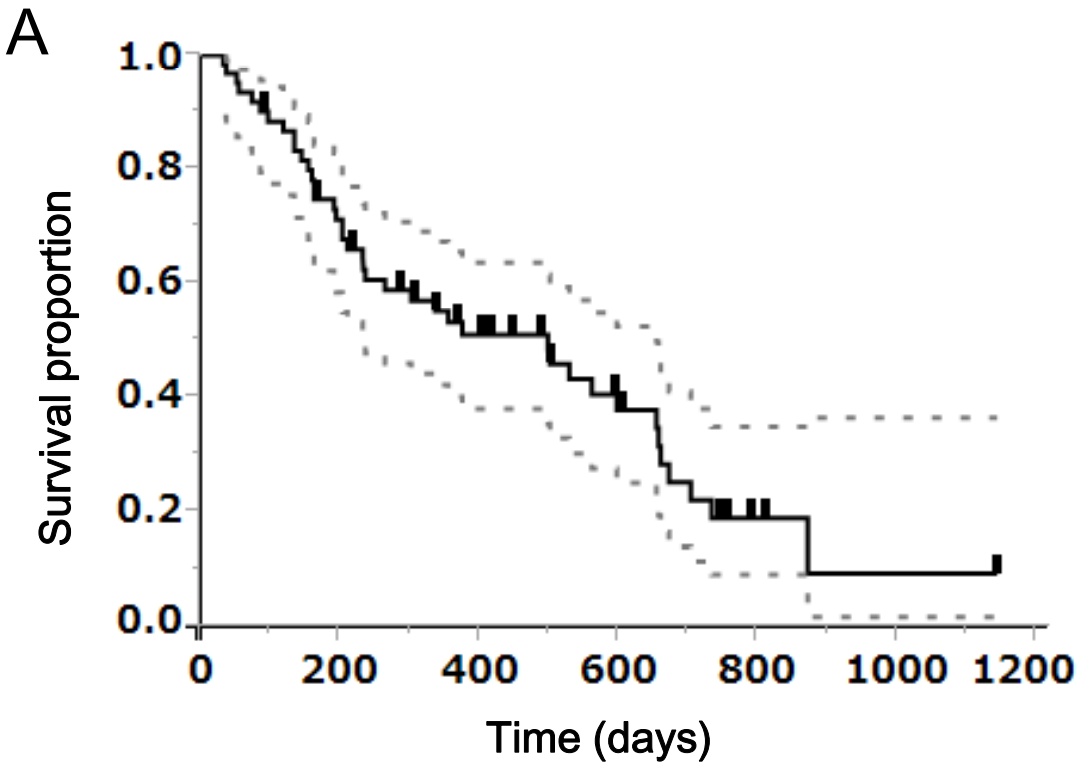
To examine the prognostic significance of CRP 1846C>T and IL-6R 48892A>C polymorphisms in aCRC patients treated with PPV, curves for overall survival (OS) were estimated by the Kaplan-Meier method, and differences between survival curves were statistically analyzed using the log-rank test. Censored patients are shown as vertical bars. (A) Patients treated with PPV were divided into three subgroups according to CRP 1846C>T polymorphisms [CRP 1846C/C (n = 10), C/T (n = 23), T/T (n = 27)]. (B) Patients treated with PPV were divided into two subgroups according to the IL-6R 48892A>C polymorphisms [IL-6R 48892C/C or A/C (n = 40) vs IL-6R 48892A/A (n = 20)]. (C) Patients treated with PPV were divided into four subgroups according to the IL-6R

48892A>C polymorphisms (IL-6R 48892C/C or A/C vs IL-6R 48892A/A) and IL-6 levels (negative or positive) in pre-vaccination plasma [IL-6 (-), IL-6R C/C or A/C (n = 18); IL-6 (-), IL-6R A/A (n = 11); IL-6 (+), IL-6R C/C or A/C (n = 21); IL-6 (+), IL-6R A/A (n = 9)].

Figure 3. Prognostic significance of increased peptide-specific CTL or IgG responses in aCRC patients after PPV.

CTL and IgG responses specific to the vaccine peptides were determined by IFNγ ELISPOT and bead-based multiplex assays, respectively. (A) A representative result of IFNγ ELISPOT assay with PBMCs stimulated with control (HIV-derived peptide) or vaccine peptides is shown before and after vaccination (Patient #4). Average spot numbers of triplicate wells are shown. (B and C) Patients treated with PPV were divided into two subgroups according to the presence or absence of increased peptide-specific CTL responses (B) or IgG responses (C) after the first cycle of vaccination. Curves for OS were estimated by the Kaplan-Meier method, and a difference between survival curves was statistically analyzed by the log-rank test. Censored patients are shown as vertical bars.

Figure 1


A
1.0
0.8
0.6
0.4
0.2
0.0
Survival proportion
0
200
400
600
800
1000
1200
Time (days)

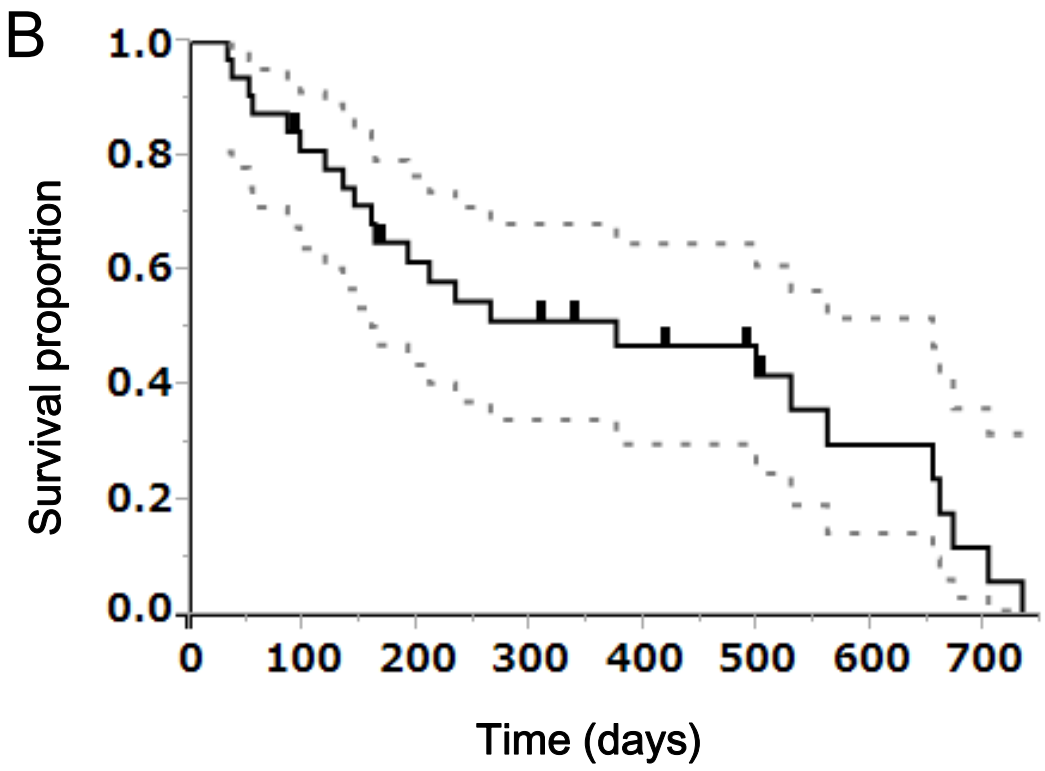

B
1.0
0.8
0.6
0.4
0.2
0.0
Survival proportion
0
100
200
300
400
500
600
700
Time (days)

Figure 2

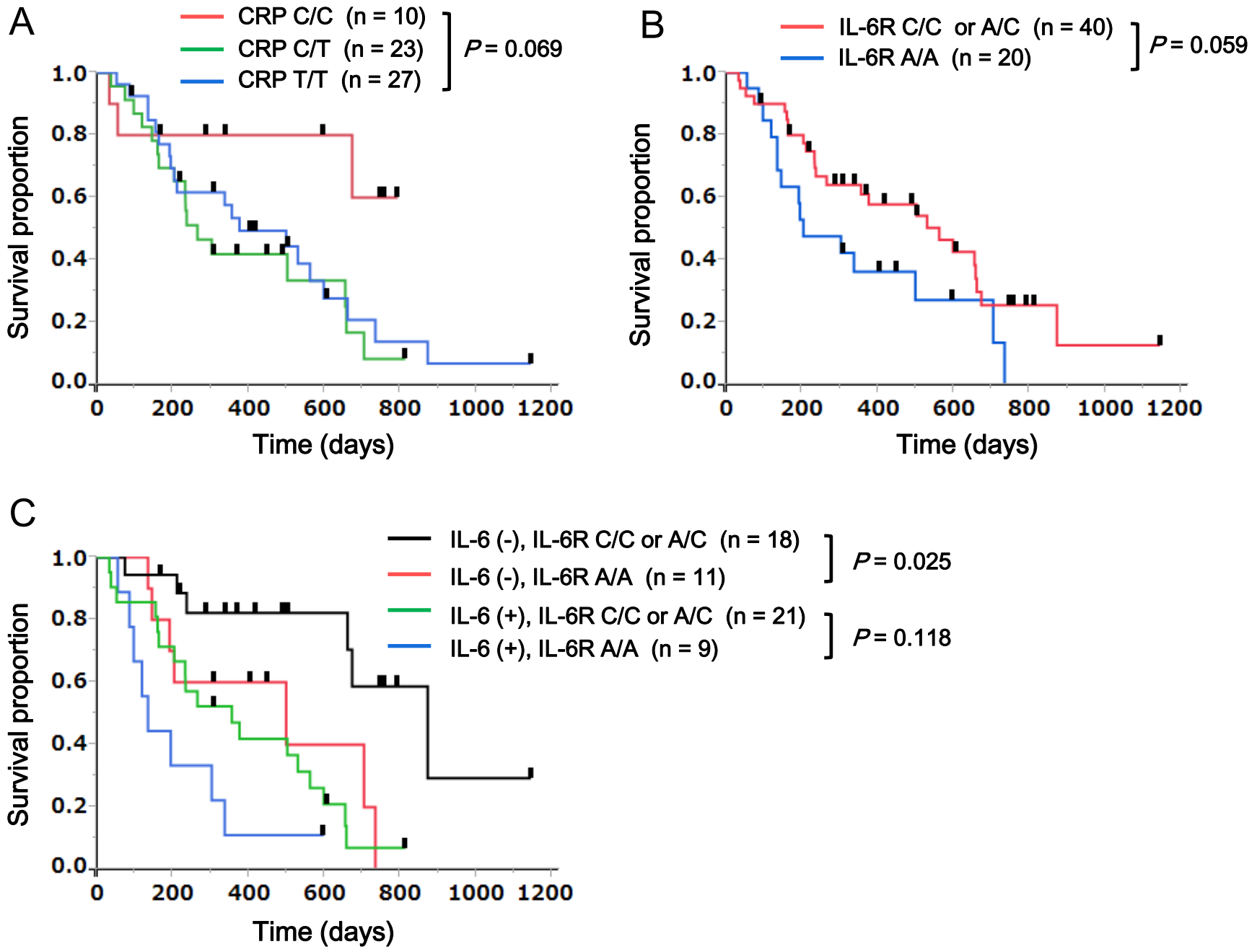
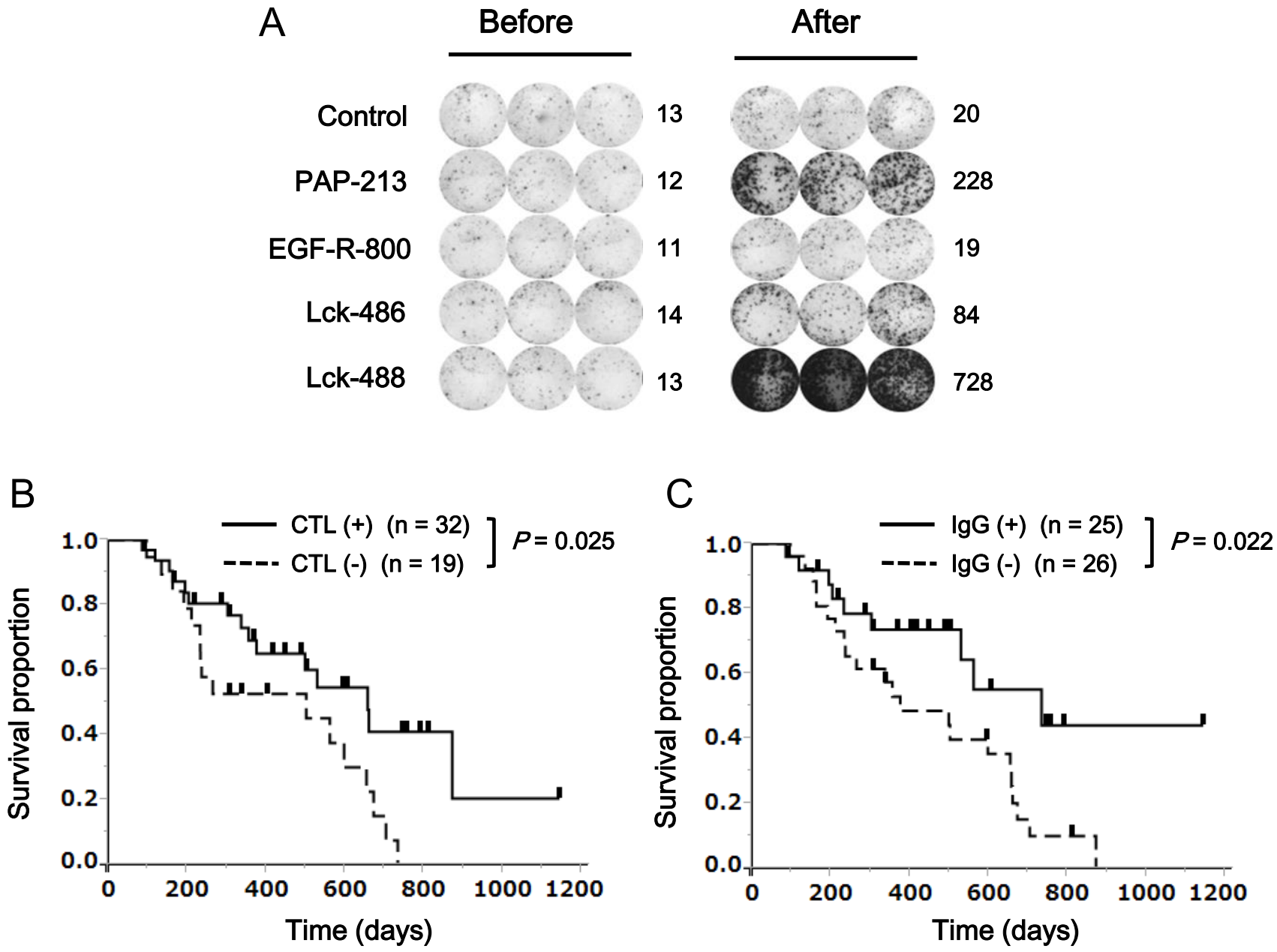

A
CRP C/C (n = 10)
CRP C/T (n = 23)
CRP T/T (n = 27)
P = 0.069
Survival proportion
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
Time (days)
B
IL-6R C/C or A/C (n = 40)
IL-6R A/A (n = 20)
P = 0.059
Survival proportion
Time (days)
C
IL-6 (-), IL-6R C/C or A/C (n = 18)
IL-6 (-), IL-6R A/A (n = 11)
IL-6 (+), IL-6R C/C or A/C (n = 21)
IL-6 (+), IL-6R A/A (n = 9)
P = 0.025
P = 0.118
Survival proportion
Time (days)

Figure 3


A
Before
After
Control
PAP-213
EGF-R-800
Lck-486
Lck-488
13
12
11
14
13
20
228
19
84
728
B
CTL (+) (n = 32)
CTL (-) (n = 19)
P = 0.025
Survival proportion
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
Time (days)
C
IgG (+) (n = 25)
IgG (-) (n = 26)
P = 0.022
Survival proportion
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
Time (days)