Original Paper

Prognostic Factors in Gastrointestinal Perforation

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KEY WORDS: Gastrointestinal perforation, Prognosis, Complications.

ABBREVIATIONS: Gastrointestinal (GI); Disseminated Intravascular Coagulation (DIC); Sequential Organ Failure Assessment (SOFA); Acute Lung Injury (ALI); Acute Respiratory Distress Syndrome (ARDS); Systemic Inflammatory Response Syndrome (SIRS).

ABSTRACT

Background/Aims: Postoperative complications associated with gastrointestinal (GI) perforation may lead to a poor prognosis. The goal of the study was to identify factors required for the establishment of appropriate perioperative procedures in such cases.

Methodology: The subjects were 51 patients with GI perforation treated from July 2007 to June 2008 in six hospitals in the Minamikawachi district.

Results: The perforation sites were the large intestine in 22 cases, small intestine in 15, stomach in 7 and duodenum in 7. Postoperative complications developed in 25 cases (49%), including infection in 20 and respiratory dysfunction in 13. Hospital mortality was 25% and the major causes of death were infection and respiratory dysfunction. The mortality was 52% and 0% in patients with and without postoperative complications, respectively. The mortality was 69% in the 13 patients with postoperative respiratory dysfunction compared to 11% for patients without respiratory dysfunction. Of the 7 patients with large intestine perforation, 4 were treated with sivelestat sodium. These 4 patients had a high mean SOFA score (11.5±1.3), but 2 out of 4 survived.

Conclusions: Postoperative complications occurred in approximately half of the patients with GI perforation and were associated with a poor prognosis. Prevention of respiratory dysfunction is particularly important for an improvement of outcome.

INTRODUCTION

Gastrointestinal (GI) perforation is associated with complications after surgery because the abdominal cavity is likely to be contaminated with GI contents and enteric bacteria (1). Complications range from local disorders such as surgical wound infection to systemic complaints including shock, sepsis, respiratory dysfunction and disseminated intravascular coagulation (DIC). These problems may result in an unexpected deterioration in the systemic condition from which it is difficult for the patient to recover. Therefore, we investigated the patterns and incidences of complications during and after surgery for GI perforation, with the goal of identifying prognostic factors for the establishment of appropriate procedures for perioperative care.

METHODOLOGY

The study was performed retrospectively in patients with GI perforation who were treated from July 2007 to June 2008 in six hospitals in Osaka and Wakayama. These hospitals are members of the Minamikawachi Study Group for Perioperative Care (**Table 1**). Patients with GI perforations due to appendicitis and anastomotic leaks were excluded from the study. The patients' background, postoperative complications, onset date of complication, outcome and sequential organ failure assessment (SOFA) score were investigated. Survival during hospitalization was used as the outcome. Organ dysfunction was defined based on a SOFA score ≥2 in each organ. Statistical analysis was conducted using a Fisher exact test with the significance level set at 0.05. Values in figures and tables are presented as means ± standard deviation.

RESULTS

Patient background and characteristics

Fifty-one patients with GI perforation were treated in the 6 hospitals over the study period of one year. The background of these patients is shown in **Table 2**. The patients included 30 males and 21 females, and had a mean age of 66.2±14.7 years. The major perforation site was the large in-

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TABLE 1 Member Institutions of the Minamikawachi Study Group for Perioperative Care

Institution
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Department of Surgery, Minamiwakayama Medical Center, National Hospital Organization
Department of Gastrointestinal Surgery, Shiroyama Hospital, Healthcare Corporation Shunju-kai
Department of Surgery, PL Hospital, Healthcare Corporation Hosho-kai
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testine in 22 patients, the small intestine in 15, the stomach in 7, and the duodenum in 7. The mean time from onset to surgery, the mean hospitalization period, and the mean ICU stay were 23.7±27.9 hours, 54.2±55.1 days, and 2.3±5.0 days, respectively. There were considerable individual differences in all three of these indicators; however, the hospitalization period showed a tendency to be prolonged in patients with perforation of the large and small intestines (data not shown).

Postoperative complications

Twenty-five (49%) out of the 51 patients had operative complications (**Figure 1**). The major complication was infection (including surgical wound infection) in 20 patients, followed by respiratory dysfunction such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in 13 patients. Other complications were DIC in 6 patients, shock in 6, liver damage in 5, anastomotic leak in 4, kidney damage in 3, and others in 4. The incidence of complications by perforation site was 12 (46%) in the large intestine, 10 (67%) in the small intestine, 2 (29%) in the stomach and 1 (14%) in the duodenum.

Outcomes

Thirteen (25%) out of the 51 patients died in hospital (**Figure 2**), including 6 patients with a large intestine perforation, 5 with a small intestine perforation and 2 with a stomach perforation. There were no deaths among patients with perforation of the duodenum. The major cause of death was infection (10 patients), followed by respiratory dysfunction (9 patients). Thirteen (52%) of the 25 patients with postoperative complications died, but all 26 patients without postoperative complications survived, with a significant difference between the two groups (**Figure 2**). Of the 20 patients with postoperative infection, the infection was limited to the surgical wound in 13 cases and 2 (15%) of these patients died. In the other 7 patients, the infection had spread beyond the surgical wound (intra-abdominal infection: 4; sepsis: 2; gallbladder infection: 1) and all 7 patients died (**Figure 3**). Of the 13 patients with respiratory dysfunction, 9 (69%) died, whereas only 4 (11%) of the 38 patients without respiratory dysfunction died. There was a significant difference in outcomes between these groups. Patients with respiratory dysfunction had a significantly higher incidence of infection, DIC, liver and kidney damage and shock compared to those without respiratory dysfunction (**Table 3**).

Period to onset of organ dysfunction

The period to onset of organ dysfunction was assessed using the SOFA score. The mean period was

TABLE 2 Background Data

Item		Value
Number of patients		51
Gender	Male	30
	Female	21
Age (years)		66.2±14.7 [14-90]
Perforation site	Large intestine	22 (43%)
	Small intestine	15 (29%)
	Stomach	7 (14%)
	Duodenal intestine	7 (14%)
Time from onset to surgery (h)		23.7±27.9* [0-120]
Hospitalization period (days)		54.2±55.1* [10-294]
ICU-stay period (days)		2.3±5.0 [0-28]

Data are shown as the mean ± S.D. with the minimum – maximum range in parentheses
* Excluding one patient for whom data were unavailable.

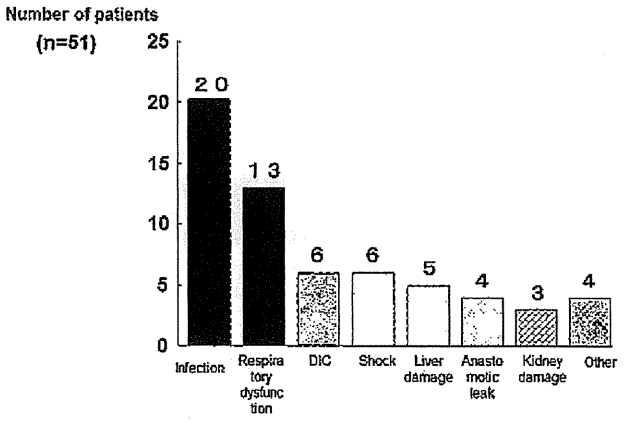

FIGURE 1 Postoperative complications. The number of subjects with each complication is shown. Some patients had multiple complications. Ileus, venous thrombosis, impaired consciousness and small intestine perforation each occurred in one patient.

the shortest in cases with respiratory dysfunction (0.25±0.46 days), followed by cardiovascular, kidney, liver and central nervous system diseases. The longest onset period was 3.00±1.22 days in cases with DIC (Figure 4).

Effect of sivelestat sodium

The mean SOFA score was 8.0±4.5 in 7 patients with large intestine perforation for whom this score was available. Four of these patients were given sivelestat sodium. These 4 patients had a higher mean SOFA score (11.5±1.3) than the other 3 patients (3.3±1.5), but 2 of the 4 patients survived, compared to only 1 survivor among the 3 patients who were not treated with sivelestat (Figure 5).

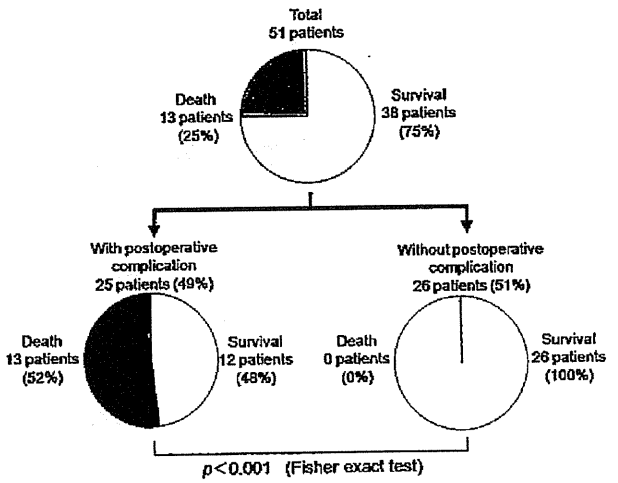

FIGURE 2 Overall outcomes in the 51 patients in the study.

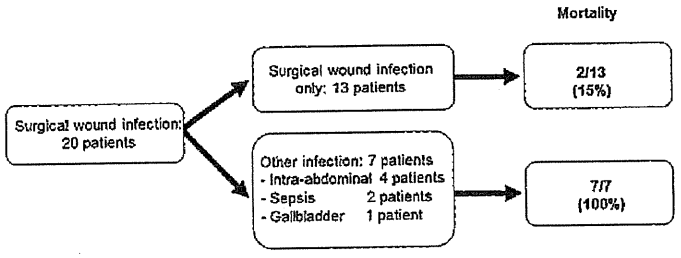

FIGURE 3 Outcomes in 20 patients with postoperative infection.

TABLE 3 Outcomes for Patients with (n=13) and without (n=38) Respiratory Dysfunction

Outcome	Respiratory dysfunction	No respiratory dysfunction	*p*-value
Death	9 (69%)	4 (11%)	0.0001
Infection	11 (85%)	11 (29%)	0.0008
DIC	5 (38%)	1 (3%)	0.0028
Liver damage	4 (31%)	1 (3%)	0.0121
Kidney damage	3 (23%)	0 (0%)	0.0137
Shock	4 (31%)	2 (5%)	0.0307

DISCUSSION

Postoperative complications associated with GI perforation include local disorders such as surgical wound infection and systemic disorders that are more difficult to treat (1). Such postoperative complications worsen the prognosis (2) and in this study we also found that patients with postoperative complications had a poorer prognosis than those without complications. The exact incidence of postoperative complications associated with GI perforation is unclear, but several studies have found a complication rate of 60%-80% (3-6). However, many of the subjects in these studies were patients with lower GI perforation and many were also elderly. In our study, the incidence of postoperative complications was 52%. However, given the short hospitalization period and short-term nature of the study, this is still an unacceptable rate. In addition, it has been found that patients with large intestine perforation who develop multiple organ dysfunction have poor long-term outcomes (7). Therefore, further follow-ups of the patients in this study are required to obtain a full assessment of the outcome.

Infection was the most frequent postoperative complication among our patients, with an incidence of 39.2% (20/51 cases). Patients with infection at a site other than the surgical wound had a particularly poor prognosis, which suggests that perioperative procedures are required to prevent infection of other organs and to treat patients with this type of infection. Postoperative complications associated with GI perforation are likely to occur in distant organs, and these complications can develop even with appropriate surgery and postoperative care including measures against infection. These complications are caused by the release of bacteria into the abdominal cavity from the intestine, which stimulates production of inflammatory cytokines that induce systemic inflammatory response syndrome (SIRS) (8), activate neutrophils and stimulate endothelial cells. This leads to systemic distribution of mediators including neutrophil elastase and oxygen radicals released from activated neutrophils, and eicosanoids and nitric oxide produced in vascular endothelial cells. This establishes a vicious cycle in which these mediators further increase inflammatory cytokine production, resulting in postoperative complications such as shock, sepsis, respiratory dysfunction and DIC (9). Respiratory organs are particularly likely to be damaged in this situation.

Okubo *et al.* (7) found that respiratory dysfunction occurred in 15 out of 36 patients and was second only to surgical wound infection among postoperative complications in elderly patients with generalized peritonitis. Matoba *et al.* (10) showed that lung injury developed most frequently in septic multiple organ failure and was likely to develop earlier than other organ dysfunctions. Since respiratory dysfunction is one of the major dysfunctions of dis-

tant organs that can be triggered by GI perforation, it is thought to be a warning sign for other organ dysfunction. The results of this study also showed that respiratory dysfunction was the second most common complication after infection, and most of the patients with respiratory dysfunction also had complications such as DIC, liver and kidney damage, and shock. In a small number of patients in whom the onset of organ dysfunction was shown by the SOFA score, respiratory dysfunction had a tendency to develop earlier than other organ dysfunction. Based on these results, we suggest that a key for improving the prognosis of patients with GI perforation is to prevent respiratory dysfunction or to prevent other organ dysfunction by intensive treatment if a patient does develop respiratory dysfunction after surgery.

Large intestine perforation is likely to result in a severe status in comparison with other GI perforation and the mortality is high (1). Komatsu *et al.* (11) proposed that a SOFA score ≥8 is a predictive factor for a poor prognosis. In our study, the SOFA score was available for 7 patients with large intestine perforation. These patients had a mean score of 8.0±4.5 and 3 (43%) died. Sivelestat sodium has a selective inhibitory effect on neutrophil elastase and is indicated for acute lung injury associated with SIRS (12,13). Many studies have shown that sivelestat is also effective against dysfunction of organs other than the lung. Four of the patients with large intestine perforation were treated with sivelestat sodium. This group had a mean SOFA score of 11.5±1.3, which suggests that they had severe conditions, but two of these patients survived. This suggests that treatment with sivelestat may improve the prognosis of patients with large intestine perforation. However, the number of subjects was small and the dose and administration period were variable; therefore, further studies are required to draw a clearer conclusion.

In conclusion, postoperative complications associated with GI perforation were found in approximately half of the patients with GI perforation in Minamikawachi district. Postoperative complications were associated with a poor prognosis and infection of regions beyond the surgical wound and respiratory complications had particularly adverse effects on prognosis. Sivelestat sodium may be useful for the improvement of outcome after surgery for GI perforation and perioperative measures to prevent respiratory dysfunction are also likely to improve the prognosis.

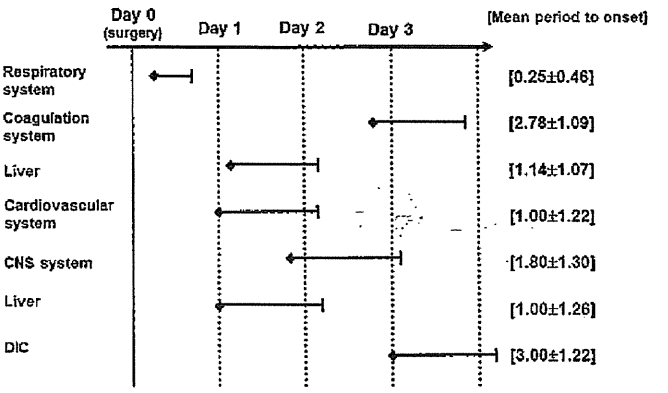

FIGURE 4 Period to onset of organ dysfunction in 12 patients.

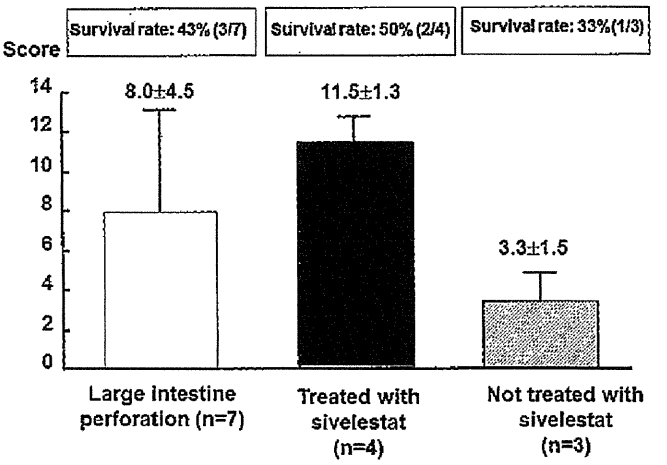

FIGURE 5 Preoperative SOFA scores in 7 patients with large intestine perforation.

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Phase I clinical trial of a novel peptide vaccine in combination with UFT/LV for metastatic colorectal cancer

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Abstract. To test the safety and immune responses of a novel peptide vaccine derived from RNF43 (ring finger protein 43) and TOMM34 (34-kDa translocase of the outer mitochondrial membrane) administered in combination with chemotherapy in patients with metastatic colorectal cancer, a phase I clinical trial with 21 HLA-A2402-positive metastatic colorectal cancer patients was conducted. Patients received a weekly peptide vaccine (1 mg of each peptide in incomplete Freund's adjuvant) in combination with oral UFT (300 mg/m^2/day) and UZEL (75 mg/day) for 4 weeks, followed by 1 week of rest. The protocol consisted of at least two cycles of this regimen. After the 2nd cycle, vaccinations were given biweekly or monthly, depending on the condition of the patient. Clinical responses were judged 10 weeks after the 2nd cycle by performing computed tomography (CT) scans and assessing the cytotoxic T lymphocyte (CTL) responses against RNF43 and TOMM34 in peripheral lymphocytes. The vaccinations were well tolerated without any serious adverse events. CTL responses were induced against both antigens in 8 patients and against one antigen in 12 patients, while 1 patient had no CTL response. The rate of stable disease was 83%. The group with CTL responses against both antigens had the most long-term survivors, followed by the group showing CTL responses against one antigen (p=0.0079). The patients with no CTL responses had the lowest survival. The safety and immunological responsiveness of the present combination therapy suggests that it is clinically beneficial for metastatic colorectal cancer. Further clinical trials are warranted.

Introduction

Genes that are frequently up-regulated in colorectal cancer (CRC) can be identified by genome-wide analysis with cDNA microarray profiling. This strategy has been used to identify gene products that are essential for the proliferation and/or survival of CRC cells (1). Two novel tumor-associated antigens (TAAs), RNF43 (ring finger protein 43) and TOMM34 (34-kDa translocase of the outer mitochondrial membrane), were found to be up-regulated in more than 80% of CRC tissues as compared to the corresponding noncancerous mucosa (2,4). RNF43 expression cannot be detected in normal human adult organs with Northern blotting. Thus, the function of RNF43 has been associated with the proliferation of tumor cells. Since suppression of TOMM34 by siRNA was found to markedly reduce the growth of colon cancer cells, the gene product is a potential therapeutic target for human CRC (3). HLA-A24-restricted epitope peptides from RNF43 and TOMM34 for cancer vaccination for CRC patients were recently identified (2,4).

We previously reported a phase I trial involving vaccination with cancer peptides in combination with UFT and LV (UZEL) for advanced CRC patients (5). UFT is an oral anticancer drug consisting of tegafur (FT), a prodrug of 5-fluorouracil (5-FU) and uracil, an inhibitor of 5-FU degradation. LV is an oral drug consisting of calcium folinate which modulates 5-FU. We previously demonstrated that the standard dose of UFT and LV did not impede the immunological responses of advanced CRC patients to the peptide vaccination.

To investigate the safety and immunological responses of a peptide vaccination with RNF43 and TOMM34 in combination with UFT and LV, we conducted a phase I clinical study involving patients with metastatic CRC.

Materials and methods

Patients and eligibility criteria. The study protocol was approved by the Institutional Ethics Review Boards of Kinki University (approval no. 18-15) and was registered in the UMIN Clinical Trials Registry as UMIN000003728 (http://www.umin.ac.jp/ctr/index.htm). Complete written informed consent was obtained from the patients at the time of enrollment. The patients (n=23) had histologically confirmed metastatic CRC unsuitable for surgical resection and were HLA-A*2402-positive. A total of 19 patients failed to respond to prior standard chemotherapy, and the remaining 4 patients agreed to receive this immunochemotherapy (Table I). Patients were required to have completed prior chemotherapy at least 4 weeks before trial enrollment and to have

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Key words: peptide vaccine, metastatic colorectal cancer, cytotoxic T lymphocytes, immunochemotherapy

fully recovered from any adverse event with a toxicity of grade 3 or higher according to the Common Terminology Criteria for Adverse Events (CTCAE) scale. The patients were required to have an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, to be older than 20 years of age and to have a life expectancy of at least 3 months. Adequate bone marrow (white blood cell count ≥3,000/mm^3, hemoglobin ≥10 g/dl and platelet count ≥75,000/mm^3), renal function (serum creatinine ≤1.4 mg/dl) and liver function (bilirubin ≤1.5 mg/dl and transaminase within 2.5 times the institution's upper limit of normal) were required. Patients were excluded if they were pregnant or had hepatitis B or C virus antigens or human immunodeficiency virus (HIV).

Peptides. The RNF43-721 (NSQPVWLCL) and TOMM34-299 (KLRQEVKQNL) peptides were synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA) according to a standard solid-phase synthesis method and purified by reverse-phase high performance liquid chromatography (HPLC) (4,6). The purity (>95%) and the identity of the peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. RNF43-721, TOMM34-299 and the epitope peptide derived from the human immunodeficiency virus-envelope (HIV-Env) protein restricted with HLA-A*2402 (RYLRDQQLL) were used to measure the cytotoxic T lymphocyte (CTL) response.

Clinical protocol. The present open-label phase I study involved a vaccine consisting of two peptides (1 mg of each peptide) derived from RNF43 and TOMM34 mixed with incomplete Freund's adjuvant (IFA) and Montanide ISA 51 (Seppic) administered to patients with locally advanced, recurrent, or metastatic colorectal cancer. The patients received a subcutaneous injection of vaccine into the thigh or back once a week for 5 weeks. Simultaneously, patients received orally administered UFT (300 mg/m^2/day) and UZEL® (75 mg/day) for 4 weeks, followed by 1 week of rest (one cycle). The immunological responses to the inoculated peptides and clinical responses were examined after every five vaccinations. The protocol consisted of two cycles. After the second cycle, vaccinations were given biweekly or monthly (depending on patient condition), while UFT/UZEL administration was continued for 4 weeks followed by a 1-week rest period during the entire treatment period. A complete blood count and results of serum chemistry tests were obtained every 2 weeks. Clinical responses were evaluated at the end of every cycle by examining computed tomography (CT) scans and tumor markers. The vaccinated patients (n=21) were assessed for immunological and clinical responses according to the Response Evaluation Criteria in Solid Tumors (RECIST). Signs of toxicity were assessed according to CTCAE version 3.0. Overall survival rates were analyzed by the Kaplan-Meier method, and survival was measured in days from the first vaccination to succumbing to the disease. p-values were assessed using a log-rank test.

Cells. TISI cells and HLA-A*2402-positive B-lymphoblastoid cell lines were purchased from the IHWG Cell and Gene Bank (IHW no. 9042; Seattle, WA, USA) in November 2008 and stored at -80°C. Within 2 months of purchase, the cells were resuscitated and maintained in RPMI supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified 5% CO_2 incubator at 37°C. The peripheral blood was periodically collected from the enrolled patients. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and density gradient centrifugation and were frozen immediately after isolation. PBMCs from each patient were simultaneously thawed and used to measure the CTL response.

Enzyme-linked immunospot assay. For detecting antigen-specific immune responses, enzyme-linked immunospot (ELISPOT) assays were performed with the human γ-interferon (IFN-γ) ELISPOT kit (Mabtech, Nacka Strand, Sweden). Plates with 96 wells and nitrocellulose membranes (Millipore, Molshelm, France) were precoated with primary anti-IFN-γ antibody (1-D1K) at 4°C overnight.

Measurement of the cytotoxic T lymphocyte response. The IFN-γ ELISPOT assay was performed to measure the specific CTL response against the peptide. PBMCs were obtained from patients and frozen prior to vaccination and at the end of each treatment course. The frozen PBMCs were thawed and *in vitro* sensitization was performed. In brief, PBMCs were stimulated with 10 μg/ml of each peptide and 20 IU/ml of interleukin (IL)-2 at 37°C, in 5% CO_2 for 2 weeks. Peptides were added on day 0 and 7. Following incubation, the harvested cells were used as responder cells, and RNF43-721 or TOMM34-299 peptide-pulsed TISI cells were used as stimulator cells (10^5 cells per well). The HLA-A*2402-restricted epitope peptide derived from the HIV-Env protein was used as a control peptide. The IFN-γ ELISPOT kit and the AEC substrate set (BD Biosciences Pharmingen, San Diego, CA, USA) were used to measure the CTL response. Spots were captured and analyzed using an automated ELISPOT reader, ImmunoSPOT 4S (CTL Ltd., Cleveland, OH, USA). The ELISPOT assays were performed in triplicate wells. The number of peptide-specific spots was calculated by subtracting the number of spots when stimulated with the HIV-Env peptide from the number of spots when stimulated with the RNF43-721 or TOMM34-299 peptide. The percentage of specific spots was calculated by dividing the number of peptide-specific spots by the number of spots when stimulated with the RNF43-721 or TOMM34-299 peptide. CTL induction was defined as positive when more than 10 specific spots were detected or the percentage of specific spots was greater than 5%. The number of peptide-specific spots was detected as the responder/stimulator ratio-dependency.

Statistical analysis. Overall survival rates were analyzed by the Kaplan-Meier method, and survival was calculated in days from the first vaccination to succumbing to the disease. The statistical analyses were performed with SPSS statistics 17.0 (SPSS, Chicago, IL, USA).

Results

Characteristics of the patients and vaccinations. Between January 2007 and June 2009, 23 HLA-A*2402-positive patients with metastatic colorectal cancer were enrolled in the present trial. All the patients had one or more metastatic

Table I. Patient characteristics.

Patient no.	Age	Gender	Primary cancer	Sites of metastases	PS	Previous treatment
1	56	M	R	Pelvis	0	UFT, CPT-11
2	64	F	S	Lung	0	5-FU, UFT/LV
3	57	F	R	Lymph nodes	1	5-FU/LV, CPT-11, S-1
4	42	M	R	Pelvis	0	None
5	53	F	S	Lung	0	UFT/LV, vaccine
6	54	M	R	Lung	0	None
7	74	F	S	Lymph nodes	0	5-FU, UFT/LV
8	78	M	R	Lung, lymph nodes	1	5-FU, UFT/LV, CPT-11
9	58	M	R	Lung	1	None
10	46	M	T	Liver, lymph nodes	1	FOLFOX, FOLFIRI, vaccine
11	59	M	S	Primary cancer, liver, lymph nodes	1	FOLFIRI, FOLFOX
12	66	M	S	Lung, liver, lymph nodes	0	S-1
13	66	F	RS	Lung	0	UFT/LV
14	49	M	S	Lung, liver	0	None
15	51	F	S	Liver, lymph nodes	1	UFT/LV, CPT-11
16	66	M	R	Lung, liver, lymph nodes	1	UFT/LV
17	61	F	C	Liver, lymph nodes	1	FOLFOX+Bev, FOLFIRI+Bev
18	54	M	S	Primary cancer, liver, lymph nodes	0	FOLFOX+Bev, UFT/LV
19	83	M	S	Lung	0	UFT
20	66	M	R	Lung, pelvis, bone	0	FOLFOX+Bev, FOLFIRI+Bev
21	61	M	R	Lung, pelvis	1	FOLFOX+Bev, FOLFIRI, CPT-11+Cet
22	73	M	R	Lung, pelvis, lymph nodes	0	FOLFOX+Bev, FOLFIRI, CPT-11+Cet
23	65	M	R	Lung, pelvis	0	FOLFOX+Bev, FOLFIRI+Bev, IRIS

PS, Eastern Cooperative Oncology Group performance status; R, rectal cancer; S, sigmoid colon cancer; T, transverse colon cancer; RS, rectosigmoid cancer; C, cecal cancer; Bev, bevacizumab; Cet, cetuximab; IRIS, irinotecan+S-1.

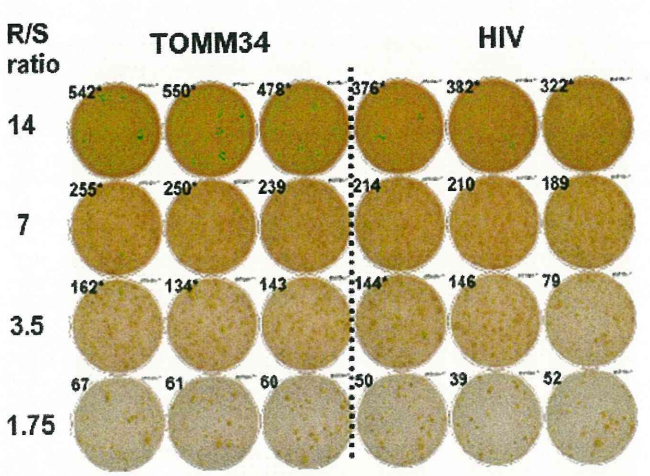
Table II. Adverse events.

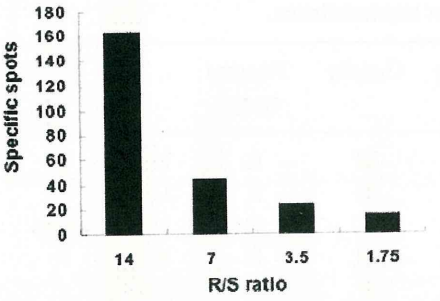
Toxicity	Total n (%)	Grade 1	Grade 2	Grade 3
Anemia	5 (23.8)	5	0	0
Transaminase elevation	3 (14.3)	3	0	0
Hyperbilirubinemia	2 (9.5)	2	0	0
Anorexia	5 (23.8)	5	0	0
Nausea	2 (9.5)	2	0	0
Malaise	3 (14.3)	3	0	0
Vaccination site reaction	15 (71.4)	15	0	0
Renal dysfunction[a]	1[a] (4.8)	0	0	1[a]

[a]A double-J catheter was placed by a urologist into one patient who experienced acute grade 3 renal dysfunction, which led to the disappearance of the hydronephrosis and the resumption of therapy.

foci that were unsuitable for surgical resection. A total of 19 patients had not responded to prior standard chemotherapy, and the remaining 4 patients agreed to receive this immunochemotherapy (Table I). A total of 2 patients (nos. 10 and 17) were disqualified as they did not meet the inclusion criteria. The final subject group thus consisted of 21 patients (15 men and 6 women) with a median age of 61 years (range 42-83). A total of 727 vaccinations were administered with a median of 31 vaccinations per patient (range 7-69). The vaccination with chemotherapy protocol was well tolerated by all patients.

Toxicities. The overall toxicities are shown in Table II. The most frequent adverse events were vaccination-site reactions (n=15), anemia (n=5), anorexia (n=5), malaise (n=3) and





R/S ratio	Average spots		Specific spots	% Specific spots
	TOMM34	HIV		
14.0	523	360	163	31
7.0	248	204	44	18
3.5	146	123	23	16
1.8	63	47	16	25

Figure 1. Enzyme-linked immunospot (ELISPOT) assays detecting TOMM34-specific T-cell activity. Peripheral blood lymphocytes collected from patient no. 5 at the end of the second course were cultured in recombinant interleukin-2 without any antigen stimulation for 14 days and subjected to the ELISPOT assay to detect the antigen-specific T-cell response induced by the vaccination.

Table III. Immunological and clinical responses.

Patient no.	No. of vaccinations	Vaccination site reaction	CTL response	Clinical response	TTP (days)	OS (days)
1	69	Ind, red	RNF, TOMM	SD	252	1226 (alive)
2	7	(-)	RNF, TOMM	-	38	1026
3	17	Ind, red	TOMM	SD	169	448
4	16	(-)	RNF, TOMM	SD	211	741
5	69	Ind, red	RNF, TOMM	SD	365	1086 (alive)
6	31	Ind	RNF, TOMM	SD	428	1054 (alive)
7	37	Ind, red	RNF, TOMM	PD	49	1012
8	8	(-)	RNF	-	36	80
9	69	Ind, red	TOMM	SD	694	904 (alive)
11	11	(-)	RNF	PD	36	183
12	29	Ind	RNF	SD	219	387
13	37	Ind	TOMM	SD	219	521
14	54	Ind	RNF, TOMM	SD	260	512 (alive)
15	22	Ind	RNF	SD	107	197 (alive)
16	16	Ind, red	TOMM	SD	73	132
18	41	Ind	TOMM	PD	70	414 (alive)
19	52	Ind, red	RNF, TOMM	SD	309	414 (alive)
20	46	Ind	RNF	SD	218	330
21	50	Red	TOMM	SD	246	365 (alive)
22	15	(-)	TOMM	SD	69	151
23	31	(-)	(-)	SD	176	288

Two patients (no.10 and 17) were disqualified for failure to meet the inclusion criteria. CTL, cytotoxic T lymphocyte; TTP, time to progression; OS, overall survival. Ind, induration; red, redness; SD, stable disease; PD, progressive disease.

elevation of serum transaminase (n=3). With the exception of one incident of grade 3 acute renal dysfunction (no. 20) due to hydronephrosis, all of the adverse events were grade 1. A double-J catheter was placed by a urologist into the patient who experienced acute renal dysfunction, which led to the disappearance of the hydronephrosis and the resumption of therapy. This patient had a large area of tumor recurrence in the pelvis prior to therapy; therefore, the renal dysfunction due to ureteral obstruction was considered to be caused by the metastasis and not related to the therapy.

Immunological monitoring. Peripheral blood lymphocytes obtained before, during, and after the vaccination periods were cultured in rIL-2 without any antigen stimulation for

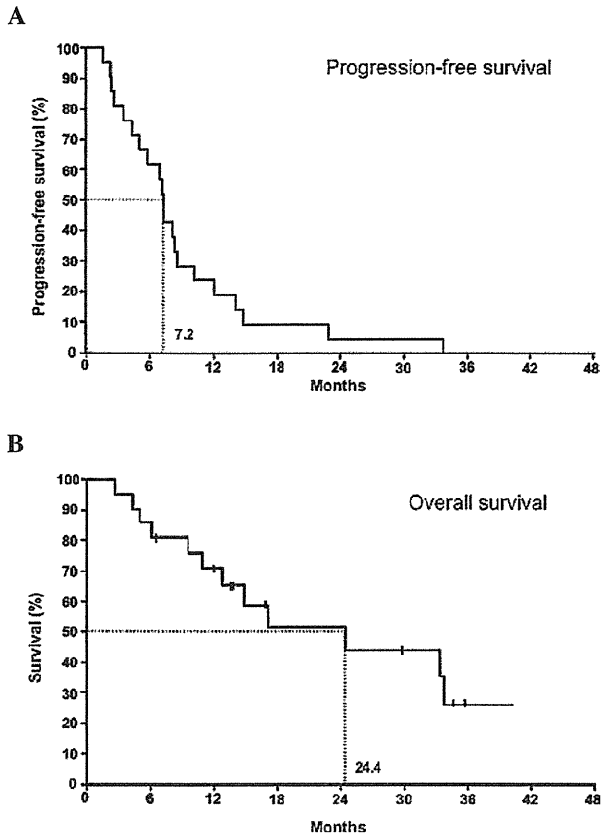


Figure 2. Survival analysis of 21 patients with metastatic colorectal cancer treated with the peptide vaccination in combination with oral chemotherapy. (A) Progression-free survival; (B) overall survival.

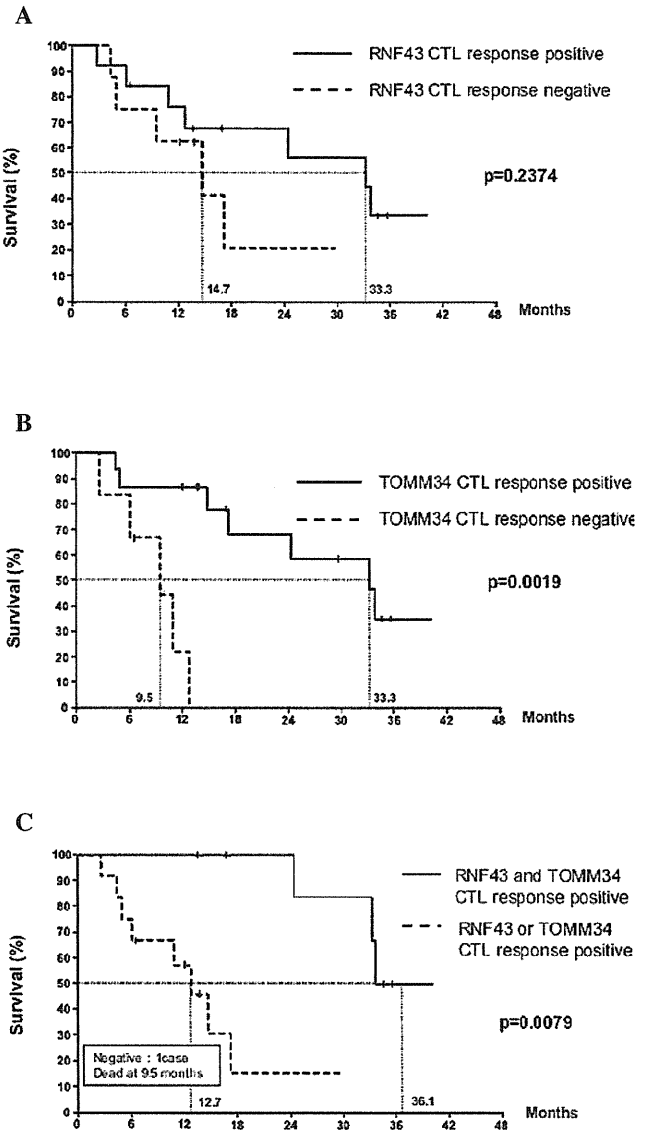


Figure 3. The relationship between cytotoxic T lymphocyte (CTL) responses and overall survival in patients with metastatic colorectal cancer treated with peptide vaccination in combination with oral chemotherapy. (A) CTL response to RNF43 and survival, (B) CTL response to TOMM34 and survival, (C) CTL responses to RNF43 and/or TOMM34 and survival.

14 days and subjected to the ELISPOT assay to detect the antigen-specific T-cell response induced by the vaccination. The CTL response was considered to be positive when more than 10 specific spots were detected or the percentage of specific spots was greater than 5%. In addition, the number of peptide-specific spots was detected as the responder/stimulator ratio-dependency. Representative CTL-positive data from ELISPOT assays against the TOMM34 antigen are shown for patient no. 5 (Fig. 1). Among the 21 patients, 8 patients had positive CTL responses against RNF43 and TOMM34, 12 patients had a positive response against one of the antigens, and the remaining patient had a negative response (Table III). The magnitude of the CTL response varied depending on the timing of the vaccinations. However, there was a clear separation between positive and negative CTL responses.

Clinical response and overall survival. Among the 21 patients, 19 patients were assessed for clinical response at the end of the 10th vaccination (2nd cycle) according to the RECIST criteria (Table III). The clinical responses of the remaining 2 patients were not assessed as they received fewer than 10 vaccinations (6 and 8, respectively). None of the patients showed a complete response or a partial response. A total of 16 patients had stable disease and 3 patients had progressive disease. The median time of progression-free survival was 7.2 months (Fig. 2A), and the mean survival time was 24.4 months (Fig. 2B).

Effect of a cytotoxic T lymphocyte response against RNF43 and TOMM34 on overall survival. The effect of a positive CTL response to RNF43 or TOMM34 on overall survival was analyzed. The Kaplan-Meier estimates for the overall survival of patients with detected CTL responses as compared to patients with no response are shown in Fig. 3. No statistical difference was found between the two groups with or without a response to RNF43 (p=0.2374) (Fig. 3A). However, there was a statistical difference between the two groups based on the TOMM34 response (p=0.0019) (Fig. 3B). Furthermore, we investigated the relationship between CTL response to both antigens and overall survival. The best long-term survival was observed in the group with CTL responses against both antigens, followed by the group showing CTL responses against only RNF43 or TOMM34 (p=0.0079). The patient with no response had the lowest survival (Fig. 3C).

Discussion

In this clinical trial, cancer vaccination with two peptides in combination with oral UFT/LV chemotherapy was well tolerated without any severe side effects in metastatic CRC patients. Common adverse events included vaccination site reaction, anemia, anorexia, malaise and elevation of transaminase. With the exception of the skin reaction, the rates of other adverse events did not exceed those of the UFT/LV chemotherapy (7). Therefore, addition of the peptide vaccination did not increase the adverse events (beyond mild vaccination site reactions) in this combination therapy. The design of this clinical trial was based on the results of two previous phase I trials. These previous trials found that vaccination with multiple peptides derived from novel cancer-testis antigens in advanced cancer was feasible and that antigen-specific T-cell responses were induced with objective clinical responses (8). These trials also showed that the peptide vaccination combined with oral UFT/LV chemotherapy was well tolerated in the metastatic CRC patients and induced peptide-specific IgG responses that correlated well with overall survival (5).

The combined chemo-immunotherapy approach has been criticized on the grounds that chemotherapy is immunosuppressive. This opinion is based on the fact that most cytotoxic drugs kill granulocyte precursors in bone marrow and thus induce leucopenia, which is associated with the occurrence of bacterial and mycotic infection. However, there is no evidence that cytotoxic chemotherapy affects the antigen-specific CTL response. Recently, Correale *et al* (9) reported that the antigen-specific killing ability of human CTL lines *in vitro* is not affected by 5-FU or oxaliplatin when exposure to these drugs does not occur during the stimulation phase. Moreover, they found that chemotherapy i) up-regulated tumor-associated antigen expression including CEA or other target molecules such as TS; ii) down-regulated tumor cell resistance to the death signals induced by tumor antigen-specific CTL; iii) reduced the percentage of PBMCs containing immune-suppressive regulatory T cells ($CD4^+CD25^+$T reg) and the number of cells expressing the FAS receptor (CD95); and iv) induced the complete restoration of the CD4/CD8 T-cell ratio, which is often reduced in advanced cancer patients resulting in a progressively deteriorating immune response (10). Based on these considerations, we believe that the rationale for chemo-immunotherapy in advanced cancer patients will be accepted.

The two cancer-specific peptides, RNF43 and TOMM34, used in the present study are novel cancer-testis antigens specific for CRC. More than 80% of colorectal cancers express these antigens, and these antigens can induce potent CTLs against colon cancer cell lines (4,6). RNF43 and TOMM34 are defined as oncoantigens. They are highly expressed in cancer cells, are involved in the critical functions of cancer cells (i.e., proliferation) and can induce potent CTL responses. In this context, it is of note that common antigens, such as MUC-1 or CEA, in colorectal cancer, are not critical for tumor cell survival; therefore, they can be lost under the selective pressure of a vaccine-induced antigen-specific immune response without significantly damaging tumor development (11-14).

Using the two crucial cancer-testis antigen-derived peptides, CTL responses were observed in 95% of the study patients (20 of 21 patients). Potent CTL responses against both antigens were induced in 8 patients (38%), and a CTL response against one peptide occurred in 12 patients (57%). Therefore, the use of two peptides allowed CTL responses to occur in almost all patients who received the vaccinations.

Overall survival was well correlated with the response to TOMM34. The patients exhibiting a response to RNF43 also experienced longer survival, although the correlation was not statistically significant. Notably, the patients exhibiting CTL responses to both peptides (n=8) had the longest survival, followed by the patients who showed a CTL response to one peptide (n=12). The patient exhibiting no response had the lowest survival (n=1) (Fig. 2). We do not have evidence to prove that the induced CTLs interacted directly with the cancer lesions in the patients with metastatic CRC to control the cancer lesions and thus contribute to the longer survival. However, we can conclude that the CTL response is a useful biomarker for patients receiving peptide vaccination therapy.

In conclusion, this study suggests that vaccination with two colorectal cancer-specific peptides in combination with UFT/LV is well tolerated and can induce potent and specific CTL responses to at least one peptide antigen in 95% of patients. Furthermore, the patients who developed potent CTL responses against both antigens showed the longest survival. This treatment approach warrants further clinical study.

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RESEARCH COMMUNICATION

Efficacy of Orally Administered *Lentinula edodes* Mycelia Extract for Advanced Gastrointestinal Cancer Patients Undergoing Cancer Chemotherapy: a Pilot Study

Kiyotaka Okuno[1]*, Kazuko Uno[2]

Abstract

This study investigated the influence of *Lentinula edodes* mycelia extract (LEM), an oral immunomodulator, on immune function and adverse events from chemotherapy. Subjects comprised 1 gastric and 7 colorectal cancer patients. The first course of treatment was chemotherapy alone and the second was chemotherapy plus concomitant administration of LEM. Adverse events and interferon (IFN)-γ production by CD4+ T, CD8+ T and CD56+ NK/NKT cells were evaluated at the end of each course. Grade 1 or 2 adverse events were observed at the end of the first course for 6 of 8 patients. In comparison, no patients displayed any adverse events at the end of the second course. Tendencies toward improved IFN-γ production by CD4+ T, CD8+ T and CD56+ NK/NKT cells were also seen. These results suggest that concomitant use of LEM with chemotherapy can decrease the incidence of adverse effects from cancer chemotherapy among patients with advanced cancer.

Keywords: *Lentinula edodes* mycelia extract - cancer chemotherapy - advanced cancer

Asian Pacific J Cancer Prev, 12, 1671-1674

Introduction

It is desirable for advanced gastrointestinal cancer patients to continue chemotherapy with fewer adverse effects as long as possible. Oral adjuvant is attractive for them because it has little burden.

Lentinula edodes has long been utilized as an edible mushroom in East Asia. A 1969 report showed that Lentinan, a high molecular weight neutral polysaccharide purified from a hot water-extract of *Lentinula edodes* (Chihara et al., 1969), has antitumor activity. Since then, Lentinan has been approved as an antitumor injection in Japan. Progress has since been made in research into the antitumor and immunomodulatory actions of the mycelia of *Lentinula edodes* (Sugano et al., 1982; Sugano et al., 1985; Liu et al., 1998) and *Lentinula edodes* mycelia extract (LEM) with hot water before germination and after culturing in a medium composed of bagasse and rice bran, is currently utilized as oral adjuvant for cancer patient .LEM has also antitumor and immunomodulatory effects(Kojima et al., 2010; Tanaka, 2011) and LEM in combination with postoperative adjuvant chemotherapy has been reported to improve the quality of life (QOL) (Nagashima,2005). In this study, the influence of LEM on immune function and adverse events resulting from cancer chemotherapy were investigated among advanced cancer patients.

Materials and Methods

Subjects

Subject in this study comprised 8 patients undergoing chemotherapy in the Department of Surgery at Kinki University between 2006 and 2007 (see Table 1). All patients showed a performance status (PS) of 0-2 and were capable of oral ingestion. All study protocols were approved by the institutional board of the Department of Surgery at Kinki University and were performed in accordance with the ethical principles designated in the Declaration of Helsinki. Prior to the study, subjects were fully informed about the objectives and methods. All

Table 1. Clinical and Other Characteristics of the Subjects in this Study

Age/Sex	Primary	Metastasis	Chemotherapy
69 f	colon	lung	irinotecan + UFT
71m	colon	liver	mitomycin C, 5-fluorouracil (HAI) + UFT
66 m	rectal, gastric	local (gast.) abdomen.	taxol
70 f	colon	liver, lung	5-fluorouracil + levofolinate + irinotecan
52 f	rectal	lung, liver	5-fluorouracil + levofolinate
60 f	rectal	pelvis	UFT
60 f	colon	lung, liver	5-fluorouracil + levofolinate

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subjects voluntarily provided written informed consent to participate in this study.

Drug

LEM was kindly provided by Kobayashi Pharmaceutical (Osaka, Japan), and was prepared as previously reported (Itoh et al., 2009).

Briefly, *Lentinula edodes* mycelia were cultivated in a solid medium composed of sugar-cane bagasse and defatted rice bran. Medium containing the mycelia was incubated in hot water, and then the soluble fraction was dried and used as LEM.

Study design

This study was conducted as an 8-week single-group open study. During the study period, each subject took two courses of chemotherapy (5-fluorouracil (5-FU), irinotecan, UFT® (uracil and tegafur), levofolinate, mitomycin or taxol). LEM was orally ingested during the second course at a dose of 1800 mg/day continuously for 4 weeks.

Tumor responses and adverse event assessments

All assessments were performed at the end of the first and second courses. Tumor responses and adverse events were evaluated according to the Response Evaluation Criteria for Solid Tumors (RECIST) and the Common Terminology Criteria for Adverse Events (CTCAE) version 2, respectively.

Cytokines and reagents

Recombinant human interleukin (rIL)-12 was provided by the Genetics Institute (Cambridge, MA). Human rIL-18 and human recombinant interferon (IFN)-γ were purchased from MBL Laboratories (Nagoya, Japan) and BD Japan (Tokyo, Japan), respectively. For cell separation, anti-CD56, anti-CD4, and anti-CD8-conjugated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used.

Preparation of peripheral blood lymphocyte (PBL) populations

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque gradient centrifugation from venous blood. PBMCs were suspended at 4 × 106 cells/ml in RPMI640 medium supplemented with 10% fetal calf serum, 5 mM Hepes and antibiotics. The suspension was cultured for 30 min in tissue culture dishes to remove adherent cells, yielding PBL populations.

Positive selection of CD56+ NK/NKT cells

CD56+ cells were separated from PBL by magnetic cell sorting using MidiMACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to a procedure that has previously been described in detail (Uno et al., 2003). The purity of CD56+ cells was in the range of 70-90% throughout this study. These cells were used as CD56+ NK/NKT cells.

Positive selection of CD8+ and CD4+ T cells

A PBL population was labeled with anti-CD56-conjugated microbeads and cleared of CD56+ cells by two passages through MidiMACSLS+ columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Contamination by CD56+ cells in the resulting CD56- population was <1%. CD56-CD8+ cells were positively separated by exposure to a magnetic field, as described previously((Uno et al., 2003)). Purity ofCD56-CD8+ cells was 95-99%. The eluted population (CD56-CD8- cells) was treated with anti-CD4-conjugated microbeads and exposed to a magnetic field. CD56-CD4+CD8- cells were positively separated (purity, 95-99%). These cells were used as CD4+ and CD8+ T-cell populations after the removal of residual macrophages by adherence to plastic.

Stimulation with rIL-12 or rIL-18

CD4+ and CD8+ T-cell populations (2×10^5 cells/well) and CD56+ cell-enriched populations (1×10^5 cells/well) suspended in complete culture medium were distributed into each well of 96-well plates at volume of 0.2 ml/well. Cells were cultured with 1000 pg/ml of rIL-12, 100 ng/ml rIL-18 or rIL-12+rIL-18 in 5% CO2 at 37°C for 20 h. Supernatants were harvested by centrifugation and stored at -80°C until use.

Measurement of IFN-γ

Concentrations of IFN-γ were measured by enzyme-linked immunosorbent assay according to a previously described procedure (Uno et al., 2003).

Statistical analysis

The measurement values are presented as mean ± standard error of the mean. Comparisons of each measurement between the two courses were analyzed using Student's paired t-test. SPSS version 13 (SPSS Japan, Tokyo, Japan) was used for all statistical analyses using a two-sided significance level of ≤5%.

Results

Adverse events

Adverse events that occurred during chemotherapy were judged using the criteria of CTCAE version 2. No adverse events assessed as grade 3 or worse occurred with any treatment. Nausea was observed in the first course (4/8 grade 1, 2/8 grade 2), along with abdominal pain (1/8 grade 1) but no adverse events were seen in the second course.

IFN-γ production by CD4+ T, CD8+ T and CD56+ NK/NKT cells

CD4+ and CD8+ T cells did not produce IFN-γ with stimulation using IL12 or IL18 alone, while IFN-γ production by CD4+ T cells stimulated using IL12+IL18 (from 471 pg/ml to 627 pg/ml), CD8+ T cells stimulated using IL12+IL18 (from 1493 pg/ml to 1735 pg/ml) and CD56+ NK/NKT cells stimulated using IL18 (from 8298 pg/ml to 9000 pg/ml) tended to increase ($p<0.1$).

Clinical findings

All patients were evaluated for response to chemotherapy with and without LEM. No patients showed marked response (complete or partial response). However,

no patients deteriorated during the treatment period. All patents were assessed as showing stable disease (SD) on computed tomography (data not shown).

Other parameters

No clinically significant differences were detected in any of the other parameters investigated.

Discussion

In this study, adverse events up to grade 2 were observed in the first course. However, no adverse events were observed in the second course. Accordingly, LEM ingestion under the conditions of this study appears to have been useful for decreasing the incidence of adverse effects caused by cancer chemotherapy.

With 5-FU, irinotecan, UFT, levofolinate, mitomycin C and taxol mono- or combination cancer chemotherapies, mild to moderate nausea and pain or other side effects are common (Gonzalez Baron et al., 1993; Naitoh et al., 1997; Takiuchi et al., 1998; Malet-Martino et al., 2002; Glimelius, 2005; Casado et al., 2008). In terms of effectiveness for patients undergoing chemotherapy, LEM has been reported to improve quality of life among breast cancer patients treated with adjuvant chemotherapy (Nagashima et al., 2005). However, no reports have clarified the effects of LEM on adverse events from chemotherapy. This is the first report on LEM to confirm effects against adverse events caused by chemotherapy. LEM has been reported to contain phenolic compounds, syringic and vanillic acid, with highly antioxidant activity (Itoh et al., 2009). Antioxidants can reportedly reduce the side effects of anti-cancer agents(Conklin, 2000), so the LEM constituents syringic and vanillic acid might have contributed to the suppression of adverse events in this study. However, LEM does not have high levels of syringic and vanillic acid contents, so components of LEM other than antioxidants seem likely to be involved in attenuating chemotherapy side effects.

In this study, grade 1 nausea and abdominal pain and grade 2 nausea were encountered, and no suspension of chemotherapy was needed in the first course. Further evaluation of LEM effects for grade 3 or worse side effects in cancer patients with chemotherapy is required.

IFN-γ production by CD4+, CD8+ T and CD56+ NK/NKT cells tended to be increased by LEM in this study. LEM reportedly acts to recover NK cell-activity suppressed by cancer chemotherapy (Nagashima et al., 2005) and β-glucan in LEM-activated antigen-presenting cells (APCs)(Kojima et al., 2010), but this is the first report the CD4+ and CD8+ T-cell activation effects of LEM in cancer patient.

Tumor-bearing patients reportedly exhibit high IL-12 or IL-18 responsiveness in T cells, and this responsiveness is induced depending on the presence of functional sets of APCs (Uno et al., 2003), so LEM might enhance CD4+ and CD8+ T-cell responsiveness by activating APCs. However, limited data were available from this study and further investigations are necessary.

In summary, ingestion of LEM decreased adverse events in a preliminary study. Ingestion of LEM appears effective for patients with advanced cancer undergoing chemotherapy. Larger-scale controlled studies are thus warranted.

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Recent Advances in Active Specific Cancer Vaccine Treatment for Colorectal Cancer

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Abstract: Cloning techniques to identify genes and peptides of tumor-associated antigens have created new possibilities for the immunotherapy of patients with advanced cancer. Here, we review recent clinical trials of specific cancer vaccines, mainly HLA-restricted peptides, and epitope-encoding vectors for advanced colorectal cancer (CRC). Many researchers initially focused on carcinoembryonic antigen (CEA) as an immunologic target antigen that is overexpressed on virtually all CRCs. A recombinant vaccine containing the CEA gene and dendritic cells (DCs) loaded with CEA peptide was administered to patients with CEA-elevated CRC. Although CEA-specific responses were detected, the clinical responses were limited. Recently, new types of clinical trials—namely, a personalized protocol to take into account the immunological diversity of cytotoxic T cell responses among patients and a novel cancer-testis antigen protocol that uses multiple peptides derived from genes identified by the cDNA array method—have been introduced. The personalized protocol seemed to be better than the classical (non-personalized) protocol in terms of clinical response and survival. Novel cancer-testis antigen protocols that use multiple CRC-derived peptides were recently conducted in patients with advanced CRC. The preliminary study yielded promising results regarding specific T cell responses to peptides and survival benefits. In this review, we summarize these results and discuss future perspectives.

Keywords: Active specific cancer vaccine, cancer-specific peptide vaccine, colorectal cancer (CRC), personalized peptide vaccine.

INTRODUCTION

Despite advances in treatment modalities, colorectal cancer (CRC) is still a leading cause of cancer-related mortality in industrialized countries. Improved treatment is urgently needed. Since the discovery of tumor-associated antigens during the early 1990s [1], rapid progress has been made in identifying antigens and describing immune interactions in cancer patients. Immunotherapeutic approaches have entered the clinical phase [2]. The goal of active specific immunotherapy is to induce an *in vivo* tumor-directed immune response. Thus, active specific immunotherapy must be distinguished from passive immunotherapy and nonspecific immunotherapy including cytokines or immunostimulants.

RATIONALE OF IMMUNOTHERAPY FOR COLORECTAL CANCER

The survival advantage of pronounced lymphocytic infiltration in CRC has been known for many years. The pioneering study by Jass showed the improved survival of CRC patients when prominent lymphocytic infiltrate was present [3]. Improved survival in patients with an increased number of peritumoral and stromal tumor-infiltrating lymphocytes (TILs) was demonstrated by Ropponen *et al.* [4]. However, in studies by Nanni *et al.* [5], Nielsen *et al.* [6], and Roncucci *et al.* [7], the number of TILs at the tumor margin or in the stroma did not influence survival in multivariate analysis. Notably, these authors did not investigate the role of intraepithelial lymphocytes (IELs). When Naito *et al.* [8] examined the role of TIL location in relation to prognosis, they found that stromal and peritumoral lymphocytes had no influence on survival, whereas the presence of IELs and CD8+ T cells in cancer cell nests was a predictor of improved outcome, independent of stage. In the same fashion, Funada *et al.* [9] demonstrated that patients with a high level of macrophage and CD8+ T cell invasion at the invasive margin had a 5-year overall survival rate of 92%, compared with a 72% survival rate in patients with a low level of infiltration Table (1).

These contradictory results may result from the complex interactions between lymphocytes, tumor, and microenvironment. It is clear, however, that the presence of activated CD8+ T lymphocytes in cancer cell nests suggests that the lymphocytes are recognizing a tumor antigen, resulting in a better prognosis.

Approximately 15% of sporadic CRCs and most hereditary nonpolyposis CRCs (HNPCCs) exhibit microsatellite instability (MSI) caused by a defect in the DNA mismatch repair system. CRCs with high MSI are usually proximal, poorly differentiated, and associated with pronounced lymphocyte infiltrate, and they have a better prognosis in comparison with MSI-negative tumors [10]. The increased im-

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Table 1. Tumor-Infiltrating Lymphocytes and Survival in CRC

Investigator	Pts N	TIL Location	RFS	OS	Follow-up
Roncucci [7] (1996)	397	Tumor margin	NR	Rectal cancer: 62% vs 36% Colon cancer: 61% vs 54%	5 Years
Ropponen [4] (1997)	195	Margin, stroma	HR, 0.72 (P<0.05)	HR, 0.55 (P<0.05)	14 Years
Naito [8] (1998)	131	Margin, stroma, IEL	NR	HR,0.91 (P=NS), HR,0.81 (N=NS), HR, 0.54 (P<0.05)	5 Years
Nielsen [6] (1999)	584	Tumor margin	NR	HR, 0.66 (P=0.03)	5 Years
Nanni [5] (2002)	263	Stroma	65% vs 58% (P=0.2)	81% vs 72% (P=0.09)	4 Years
Funada [9] (2003)	97	Margin, CD8+ T cells	NR	92% vs 72% (P<0.05)	5 Years

Studies comparing patients with colorectal cancer exhibiting prominent amounts of tumor-infiltrating lymphocytes or not and comparing of survival of CRC patients.
Pts N: patients number; TIL: tumor-infiltrating lymphocytes; IEL: intraepitherial lymphocytes; RFS: relapse-free survival; OS: overall survival; HR: hazard ratio; NR: not reported; NS: not significant

munogenicity may result from a large number of mutated proteins, which can serve as tumor-rejection antigens.

The studies described here suggest that there is a significant host response to CRCs and that the presence of the host response is associated with improved survival. These findings suggest that appropriate immunologic approaches may improve patient prognosis.

VACCINE THERAPY: SPECIFIC IMMUNOTHERAPY FOR CRC

Numerous studies have been done on vaccination in colorectal cancer patients. Among them, representative studies of antigen pulsed dendritic cells (DCs) vaccination (three studies), viral vector based vaccination (one study), personalized peptide vaccination (three studies), and colorectal cancer-specific antigen derived peptide vaccination (two studies) are summarized in Table (2).

Peptide-Pulsed Dendritic Cells

Dendritic cells (DCs) are the pivotal antigen-presenting cells (APCs) for triggering T cell immunity. Autologous DCs have been used in cancer vaccines for CRC patients. DC-based vaccines can induce tumor-specific immune responses and objective clinical responses in CRC patients with marginal adverse effects.

Liu *et al.* [11] documented an increased number of CEA-specific T cells in 7 of 10 (70%) CRC patients who received a DC vaccination. Two (20%) of these patients had stable disease for at least 12 weeks, and 1 of these 2 patients experienced a transient decrease in CEA levels during the treatment period. In a study by Weihrauch *et al.*, 17 patients received CEA-derived peptide (CAP-1) or CAP-1-pulsed DCs in combination with chemotherapy (irinotecan/ high-dose 5-fluorouracil (5-FU)/ leucovorin (LV) [12]. Five of these patients experienced a complete response, 1 patient had a partial response, 5 patients had stable disease and 6 patients had progressive disease. Favorable results may depend on concurrent chemotherapy. It is noteworthy that increases in CAP-1-specific T cells were observed in 47% of patients after vaccination, whereas the EBV/CMV recall antigen-specific CD8+ cells decreased by an average of 14% during chemotherapy. In a study by Kavanagh *et al.* [13], 13 patients with advanced CRC were treated with DCs loaded with multiple peptides derived from CEA, MAGE, and HER2/neu. When the T cell responses were examined by enzyme-linked immunospot (ELISPOT) assay, 3 patients had T cell responses to one CEA-derived peptide, and 2 patients had T cell responses to multiple peptides. However, all patients showed progressive disease.

Collectively, these results indicate that DC-based vaccination could be a promising strategy for CRC. However, multiple problems, including high cost, conflicting results, and the large amount of time required for vaccine development, must be addressed before an affordable DC-based vaccination can be developed as a standard treatment. Moreover, reliable biomarkers must be identified, and vaccines and protocols must be standardized.

Viral Vector-Based Vaccine

A recombinant vaccinia virus encoding antigen sequences, such as the CEA gene and gene products, is capable of infecting professional antigen-presenting cells (APCs) and presenting CEA peptides to T lymphocytes in the context of HLA class I and II molecules, which activate the corresponding CD8+ or CD4+ T cells. In a phase I study, the safety of the vaccine was documented, and a CEA-specific T cell response was detected; however, no significant clinical effect was observed [14]. Approaches such as boost vaccination, T cell costimulation, and granulocyte-macrophage colony-stimulating factor (GM-CSF) administration enhanced the CEA-specific T cell responses in the majority of patients [15]. A trend towards an enhanced CEA-specific immune response to vaccination and an increase in progression-free survival and overall survival was documented. However, the subject group consisted of several small cohorts with different types of cancers, including 35 CRCs and 9 lung cancers;

Table 2. Specific Vaccine Trials for Colorectal Cancer

Investigator	Vaccines	Chemotherapy	Pts N	Clinical Response	Survival
Liu [11] (2004)	DC + CEA	-	10	2 SD, 8 PD	NR
Weihrauch [12] (2005)	DC * + CEA	Irinotecan, high-dose 5-FU, LV	17	5 CR, 1 PR, 5 SD, 6 PD	OS 17mo. with survival rate of 35% (6/17)
Kavanagh [13] (2007)	DC + peptides **	-	11	11 PD	NR
Marshall [15] (2005)	Virus expressing CEA + costimulator (TRICOM)	-	35	0 CR, 0 PR	Trend towards enhanced CEA-response and an increase in PFS
Sato [18] (2004)	Peptide (personalized)	-	10	1 PR, 1 SD, 8 PD	NI
Sato [21] (2007)	Peptide (personalized)	TS-1-based	7	1 SD, 6 PD	NI, 2/7 patients still alive at follow-up (17, 30mo.)
Hattori [22] (2009)	Peptide (personalized)	UFT/LV	13	6 SD (3 MR), 7 PD	PFS 10.7 wk (range 5.0-51.0 wk), OS correlated with peptide-specific IgG
Hazama (unpublished data)	Peptides (multiple) ***	FOLFOX	26	13 PR, 12 SD, 1 PD	PFS (has not been calculated)
Okuno (unpublished data)	Peptides	UFT/LV	19	17 SD, 2 PD	PFS (7.2 mo)

*A few patients with DC; ** CEA, MAGE, HER2/neu; *** RNF43, TOMM34, KOC1, VEGFR1, VEGFR2; Pts N, patients number; NR, not reported; NI, not identifiable; SD, stable disease; PD, progressive disease; CR, complete response; PR, partial response; OS, overall survival; PFS, progression free survival

therefore, definitive conclusions regarding this method cannot de drawn.

Peptide Vaccines

Rosenberg *et al.* [16] summarized the clinical responses to peptide-based vaccine therapy in 2004. Objective response rates for peptide vaccines and viral vaccines administered to patients with metastatic cancer at the National Cancer Institute (Bethesda, Maryland, USA) were 2.9% (11 of 381 cases) and 1.9% (3 of 160 cases), respectively. In a subsequent study, those trials and other trials of cell-based therapies were analyzed collectively, giving a combined objective response rate of 3.8% (29 of 765 patients, 36 protocols). These results indicate that the classical types of cancer vaccines, including peptide vaccines, do not have a promising future as a new treatment modality for cancer.

Personalized Peptide Vaccines

In most protocols of peptide-based vaccination, no consideration has been paid to whether or not peptide-specific cytotoxic T lymphocyte (CTL) precursors are pre-existent. The initiation of immune boosting through vaccination was better than that of immune priming to induce prompt and strong immunity. Based on this concept, Itoh *et al.* [17] conducted a new regimen that included pre-vaccination measurement of peptide-specific CTL precursors in the circulation, followed by vaccination of only CTL-reactive peptide (CTL precursor-oriented vaccine). In a pilot study, 10 patients with advanced CRC were treated with up to four peptides that had been positive in the pre-vaccination measurement [18]. Post-vaccination peripheral blood mononuclear cells (PBMCs) from 5 patients demonstrated an increased peptide-specific immune response to the peptides. An increased CTL response to cancer cells was detected in post-vaccination PBMCs of 5 patients. Interestingly, antipeptide immunoglobulin G (IgG) became detectable in post-vaccination sera of 7 patients. One patient had a partial response, and another patient had stable disease for 6 months. These results are promising, but the clinical response was not satisfactory. In another protocol, the combination of this type of vaccination with chemotherapy in refractory prostate cancer patients was beneficial. This chemoimmunotherapy may break through the impasse in the clinical efficacy of cancer vaccines [19,20].

In a subsequent study, personalized peptide vaccination in combination with the oral administration of a 5-fluorouracil derivative (TS-1) in advanced CRC/ gastric cancer patients was investigated [21]. Eleven patients who did not respond to prior TS-1-based chemotherapy were enrolled. The combination therapy was generally well tolerated. The vast majority of patients experienced an increase in peptide-specific IgG after the sixth vaccination, irrespective of the dose of TS-1. In the patients who received 80 mg/m^2/day of TS-1, the CTL-mediated cytotoxicity against cancer cells was maintained at the prevaccination level. These results indicate that the standard dose (80 mg/m^2/day) of TS-1 in combination with personalized peptide vaccination does not impede immunological responses in cancer patients and could maintain or augment the immunological responses.

The combination of oral UFT® and UZEL® (LV) is a standard chemotherapy for CRC. UFT is an oral anticancer drug consisting of both Tegafur (FT), a prodrug of 5-FU, and uracil, an inhibitor of degradation of 5-FU. UZEL is an oral

drug consisting of calcium folinate, which modulates 5-FU. Therefore, we investigated the safety and immunological responses of personalized peptide vaccination in combination with UFT and LV in 14 patients with metastatic CRC [22]. Peptides were determined based on the presence of peptide-specific CTL precursors and IgG in each patient. A maximum of four peptides was administered weekly with UFT and LV for 4 weeks, followed by the standard 1-week rest period. This therapy was well tolerated, although 1 patient developed a grade 3 skin reaction at the vaccine site. After the tenth vaccination, 9 of 10 patients tested had an increase in peptide-specific interferon-γ production, and 8 of 10 patients tested had an increase in peptide-specific IgG. Six patients had stable disease, and 7 patients had progressive disease, as determined by the RECIST (Response Evaluation in Solid Tumors) criteria. Three of the 6 patients with stable disease showed a minor response; all 3 of these patients showed both strong CTL and IgG responses to at least one of the vaccinated peptides.

Interestingly, IgG responses correlated with overall survival (P= 0.0215) Fig. (**1**).

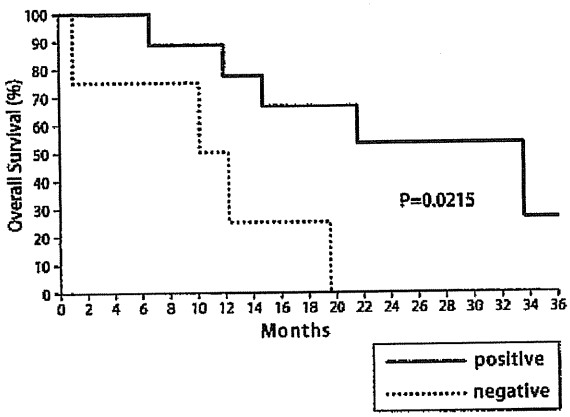

Fig. (1). Correlation between survival and peptide-specific IgG responses.
Overall survival was well correlated with increased levels of peptide-specific IgG (P=0.0215). Solid line: positive peptide-specific IgG response, dotted line: negative peptide-specific IgG response. (Ref. [22])

A similar correlation has been reported for CRC patients receiving a recombinant CEA vaccine [23]. However, the biological roles of IgGs specific to CTL epitopes are unknown. One possibility is that 9-mer peptide-recognizing CD4+ T cells were involved in this phenomenon. Peptides that bind to MHC class II molecules are generally considered to be 12 – 25 amino acids in length; however, the core sites anchored to MHC class II molecules are sufficient even at a length of about nine amino acids [24]. Indeed, our collaborator reported that the 9-mer peptide could induce peptide-specific and HLA-DR-restricted CD4+ T cells [25]. Another possibility is that CD4+ helper T cells might recognize the inoculated peptides presented on the HLA-A24 or -A2 molecules of antigen-presenting cells, resulting in both the activation of helper T cells and the subsequent promotion of IgG production [26]. CD4+ helper T cells are necessary to maintain CD8 T cell immunity [27]. If increased levels of peptide-specific IgGs reflect the activation levels of CD4+ helper T cells, the measurement of peptide-specific IgGs would be worthwhile as an immunological biomarker to predict the clinical benefits of peptide vaccination therapy for cancer patients.

In conclusion, personalized peptide vaccination combined with UFT/LV in patients with metastatic CRC is well tolerated and can induce cellular and humoral immune responses. Increased peptide-specific IgGs may be immunological biomarkers predictive of longer survival. Further trials of these vaccines are merited.

Peptides Derived from Novel Colorectal Cancer-Associated Antigens

cDNA microarray technology coupled with laser microdissection has been used to identify HLA-A24-restricted epitope peptides as potential targets for cancer vaccination in CRC patients [28, 29]. HLA-A24-positive is a dominant population in Japan (approximately 60%), subsequently HLA-A2-positive (approximately 20%). Therefore, to identify the binding epitope to HLA-A24 is essential issue for the successful anti-cancer vaccination in Japan. These antigenic peptides were derived from two different cancer-testis antigens, RNF43 (*ring finger protein 43*) [28] and TOMM34 (34 kDa-translocase of the outer mitochondrial membrane) [29]. Gene expression profiling revealed that RNF43 and TOMM34 were highly expressed in more than 80% of CRC samples, while these transcripts were hardly detectable in normal organs, with the exception of the testis and/or placenta. These peptides could stimulate CTLs that recognized and killed CRC cells. Therefore, RNF43- and TOMM34-derived peptides are promising candidates for the treatment of metastatic CRC. To evaluate the safety and immune response of vaccination with these peptides in combination with oral chemotherapy of UFT and LV for metastatic colorectal cancer, 20 HLA-A2402-positive patients were enrolled in a phase I clinical trial (Okuno *et al.* unpublished data). Eighteen patients were treated with peptides subcutaneously every week and two courses of UFT/LV chemotherapy for 10 weeks. Ten weeks later, the clinical responses were judged by CT scans, and cytotoxic T lymphocyte (CTL) responses against RNF43 and TOMM34 in peripheral lymphocytes were assessed by enzyme-linked immunospot assays. The vaccinations were well tolerated without any serious adverse events. Of the 18 patients, CTL responses were induced against both RNF43 and TOMM34 in 6 patients and against RNF43 or TOMM34 in 9 patients, while 3 patients had no CTL response. The rate of stable disease was 83%, as determined by RECIST criteria. Long-term survivors were observed in the group showing CTL responses against both RNF43 and TOMM34, followed by the group showing CTL responses against only RNF43 or TOMM34. The patients with no CTL responses had the worst survival Fig. (**2**).

Hazama *et al.* have been investigating a phase I trial of three peptides highly expressed in CRC (RNF43, TOMM34, KOC1), and the epitope peptide of vascular endothelial growth factor receptor 1(VEGFR1), vascular endothelial growth factor receptor 2 (VEGFR2) in combination with