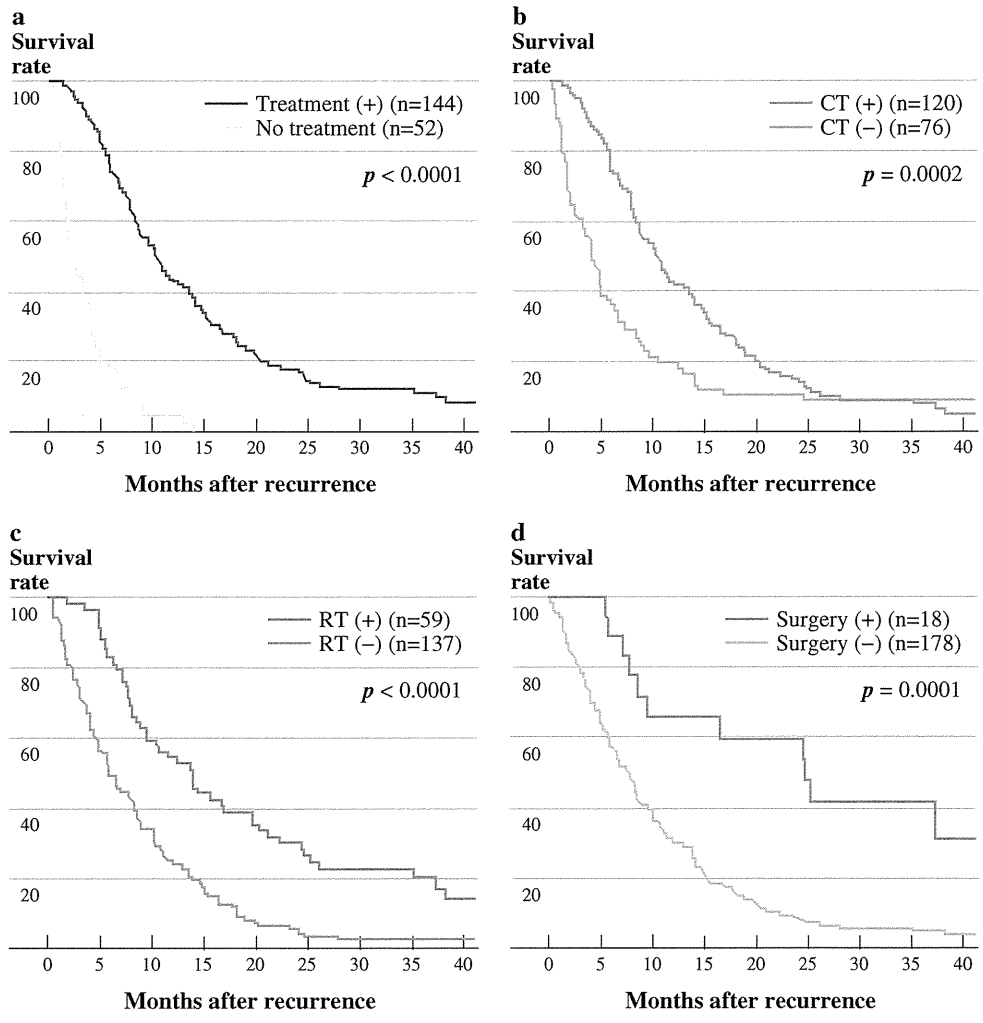

FIG. 3 Overall survival rate in 196 patients with recurrent disease after curative resection of esophageal cancer, according to treatment for recurrence. **a** Treatment versus no treatment. **b** CT versus no CT. **c** RT versus no RT. **d** surgery versus no surgery. All treatments for recurrence including chemotherapy, radiotherapy, and surgery can contribute to prolonged survival after recurrence. *CT* chemotherapy, *RT* radiation therapy

recurrence after curative resection without preoperative therapy, while Mariette et al. reported 7.0 months median survival time in patients with recurrence after surgery alone or neoadjuvant CRT followed by surgery.[3,8] In this study, median survival time after recurrence was 8.2 months. However, 66 of the 196 patients (33.7%) with recurrent disease survived for more than 1 year, and 23 (11.7%) survived for more than 2 years after recurrence. Thus, even patients who develop recurrent disease have a chance of achieving relatively long-term survival after recurrence. Moreover, the current study showed that all available treatments for recurrence including chemotherapy, radiation therapy, and surgery should prolong survival after recurrence. Thus, it is worth treating recurrent disease vigorously using available treatments as far as the patient's condition allows, although patient's condition is determined largely by the number and site of recurrent tumors.

In the present study, the recurrent disease occurred earlier in patients with advanced tumor than those with nonadvanced tumor at the time of operation (data not shown). This finding is consistent with results of previous studies.[8,28] However, the present study also showed that recurrence occurred earlier in patients who received preoperative CRT or preoperative chemotherapy than in those treated by surgery alone, although there is no significant difference in tumor stage at the time of operation (pathological tumor stage) between the former and the latter. One possible explanation for this result may be that postoperative complications is associated with early recurrence in patients who underwent esophagectomy for esophageal cancer.[29] In fact, the incidence of postoperative complication tended to be higher in patients who received preoperative CRT than those treated by surgery alone (49.4 vs. 38.3%). However, recurrence occurred earlier in patients who underwent preoperative chemotherapy than those treated by surgery alone, although there is no difference in incidence of complication between the 2 (39.4 vs. 38.3%). Another reason may be that many of patients who developed recurrent disease after preoperative chemotherapy or preoperative CRT are nonresponders in this study (data not shown). Ineffective preoperative therapy may hasten the occurrence of recurrent disease after

TABLE 4 Results of multivariate analysis for survival after recurrence

	Model A			Model B		
	HR	95% CI	*P* value	HR	95% CI	*P* value
cStage						
III/IV	1.13	0.79–1.60	0.5076	1.13	0.79–1.63	0.4948
Preoperative therapy						
CRT performed	1.64	1.08–2.48	0.0189	1.78	1.17–2.71	0.0068
pStage						
III/IV	1.41	1.01–2.04	0.0498	1.42	0.97–2.09	0.0700
Recurrence within 1 year						
Present	1.33	0.89–1.99	0.1638	1.55	1.00–2.41	0.0498
Number of recurrent tumors						
≥4	2.06	1.40–3.01	<0.0001	1.80	1.21–2.67	0.0035
Recurrence at local site						
Present	1.85	1.23–2.78	0.0032	1.38	0.91–2.09	0.1348
Recurrence in liver						
Present	1.79	1.20–2.68	0.0044	1.68	1.136–2.49	0.0107
Recurrence in bone						
Present	1.06	0.66–1.71	0.8112	1.09	0.68–1.74	0.7208
Treatment for recurrence						
CT performed				0.34	0.23–0.49	<0.0001
RT performed				0.44	0.29–0.67	0.0001
Surgery performed				0.53	0.29–1.05	0.0682

CT chemotherapy, *CRT* chemoradiation therapy, *RT* radiation therapy, *HR* hazard ratio, *95% CI* 95% confidence interval

TABLE 5 Treatment for recurrence according to number of recurrent tumors

	Number of recurrent tumors		
	1	2–3	≥4
n	74	46	76
Treatment used for recurrence			
None*	13 (18)	12 (26)	27 (36)
CT	44 (59)	28 (61)	48 (63)
RT**	35 (47)	16 (35)	8 (11)
Surgery***	14 (19)	3 (7)	1 (1)

CT chemotherapy, *RT* radiation therapy

* $P = 0.0321$; ** $P < 0.0001$; *** $P = 0.0004$

surgery rather than delay it because of suppression of immune function caused by preoperative therapy.

This retrospective study has a limitation by allowing selection bias, in that indication for initial treatment depends not only on patient selection but also on clinical tumor stage. In fact, initial tumor stage in patients who were treated with preoperative chemotherapy or CRT followed by surgery tended to be more advanced than those who underwent surgery alone. However, there was no significant difference in pathological tumor stage after treatment between patients with and without preoperative therapy, when our examination is limited to patients with recurrent disease. As shown in previous studies, whether patients develop recurrent disease after esophagectomy or not depends largely on pathological tumor stage.[3–8,28] Furthermore, in our study, multivariate analysis identified pathological tumor stage but not clinical tumor stage as an independent prognostic factor after recurrence. Therefore, we do not think that comparing the recurrence pattern and survival after recurrence between patients with and without preoperative therapy is invalid in this study.

In conclusion, our study demonstrated that among various clinicopathological factors, pathological tumor stage, preoperative CRT, the number of recurrent tumors, and recurrence at local site and liver are significantly associated with survival of patients who developed recurrence after curative resection of esophageal cancer. Although the choice of treatment option for recurrent disease is limited by the number of recurrent tumors and preoperative CRT performed, our study also revealed that all treatments for recurrence including chemotherapy, radiotherapy, and surgery could contribute to prolonged survival after recurrence. Vigorous treatment for recurrence might therefore extend survival after recurrence in patients who underwent esophagectomy.

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Minimally Invasive Esophagectomy for Esophageal Cancer: Comparative Analysis of Open and Hand-Assisted Laparoscopic Abdominal Lymphadenectomy with Gastric Conduit Reconstruction

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Background and Objectives: Esophagectomy for esophageal cancer is an invasive procedure. Minimally invasive approaches such as hand-assisted laparoscopic surgery (HALS) might reduce surgical stress and improve postoperative course.

Methods: We retrospectively analyzed 216 consecutive patients who underwent esophagectomy for esophageal cancer through either HALS (109 patients) or open laparotomy (107 patients), through an abdominal approach. The peri- and postoperative outcomes were compared between the two groups.

Results: No significant difference was observed in physical and tumor status between the two groups. The mean operating time (HALS: 452 ± 65, Open: 456 ± 69 min) and mean number of resected lymph nodes (HALS: 19.3 ± 7.1, Open: 20.8 ± 8.3) were similar, while total blood loss was lower in HALS (HALS: 695 ± 369, Open: 1,101 ± 540 ml; $P = 0.0001$). The postoperative course showed marginally lower incidences of pulmonary (HALS: 6.4%, Open: 14.0%; $P = 0.062$) and overall complications (HALS: 23.9%, Open: 35.5%; $P = 0.11$), lower C-reactive protein level at postoperative days 1, 3, and 7, and shorter duration of systemic inflammatory response syndrome (HALS: 2.3 days, Open: 3.5 days; $P = 0.0002$) in HALS than in OPEN. The disease-free survival rates at 2 years were 65% in HALS and 53% in Open.

Conclusions: The findings suggest that HALS is feasible and useful for patients with esophageal cancer.

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KEY WORDS: HALS; esophagectomy; esophageal cancer

INTRODUCTION

Radical esophagectomy currently provides the best cure for resectable esophageal cancer. However, it is a highly invasive surgical procedure associated with high morbidity and mortality. The reported postoperative morbidity rate ranges from 45% to 80%, even in high volume centers [1,2]. Especially, respiratory complications occur at a frequency of 30–75% and are often severe and potentially lethal [3,4]. Therefore, there is a need for alternative techniques that do not only have acceptable oncological outcome but also diminish surgical stress and postoperative complications.

In 1980s, laparoscopic techniques were introduced to the field of upper gastrointestinal surgery and colorectal surgery as less invasive surgery, and subsequent reports indicated that such techniques reduce postoperative pain and complications and hasten recovery [5–7]. In esophageal cancer, esophagectomy via thoracoscopic procedures was introduced in 1992, and various less invasive techniques such as thoracoscopy, laparoscopy, and their combination have since been reported [8–10]. Despite their wide use in clinical practice, the safety and efficacy of these techniques remain controversial [11,12].

Hand-assisted laparoscopic surgery (HALS) has recently gained clinical acceptance as a practical and useful alternative technique to laparoscopic and open surgery [13–16]. HALS is not only less invasive than open surgery but also causes less damage to organs and is easier than laparoscopic surgery based on its manual nature and ability to use retractors. In theory, HALS seems appropriate procedure, similar to the abdominal approach in radical esophagectomy, for patients with thoracic esophageal cancer. To date, no report has evaluated the feasibility and usefulness of HALS compared with open laparotomy.

The aim of the present study was to evaluate the feasibility and usefulness of HALS, and to compare it with the abdominal approach in radical esophagectomy. The study also compared the short-term and mid-term outcomes with those of the open procedure.

PATIENTS AND METHODS

Patients

From January 2005 to January 2010, a total of 310 patients diagnosed histopathologically with esophageal squamous cell carcinoma (ESCC) underwent surgery at Osaka University Hospital. All patients were new cases of ESCC and none had received treatment prior to surgery. All underwent esophageal fiberscopy, esophagography, and enhanced computed tomography (CT) scanning from the neck to the abdomen for tumor staging according to the 6th edition of the TNM classification of the International Union Against Cancer (UICC).

All 216 consecutive patients who met the following inclusion and exclusion criteria were enrolled in this study: (1) subtotal esophagectomy and mediastinal lymph node dissection was performed through right thoracotomy; (2) the primary tumor was located in the thoracic esophagus; (3) the reconstructive organ was the stomach but not the jejunum or colon; (4) the primary tumor or metastatic lymph nodes showed no contiguous spread to adjacent organs such as trachea, aorta, lung in the preoperative evaluation, unless these tumors were

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regarded as completely resectable; (5) no history of previous upper abdominal surgery; (6) no abdominal procedure by totally laparoscopy and/or video-assisted thoracoscopic surgery (VATS); (7) no additional abdominal para-aortic lymph nodes dissection or resection of other organs such as the gallbladder; (8) no pull-up procedure of the gastric conduit through retrosternal or subcutaneous route; and (9) lack of active malignancy in another organ. Abdominal lymphadenectomy and gastric conduit reconstruction was performed by HALS group in 109, while the remaining 106 patients underwent open laparotomy (Open group). In our institution, open laparotomy was performed prior to September 2007 while HALS was employed from October 2007 onwards. Each of the open operations were performed by four upper-GI specialists, while HALS was performed by only two (M.Y. and H.M.). In the first 2 months of the introduction of HALS, only one surgeon (M.Y.) performed HALS as the chief operator with the assistance of the other surgeon (October–November 2007).

Treatment Protocol

The basic strategy for treatment of patients with ESCC had been described previously [17]. In brief, cT4 were indicated for chemoradiotherapy as the initial treatment, and then surgical resection was performed when patients were diagnosed as downstage, release of T4. On the other hand, cN1and/or cM1lym with cT1-3 were indicated for neoadjuvant chemotherapy followed by surgery. Furthermore, cT1-3N0 was indicated for surgery without preoperative treatment between January 2005 and January 2009, and cT2-3N0 was indicated for surgery with neoadjuvant chemotherapy after January 2009. Surgery was performed 4–8 weeks after preoperative chemotherapy.

The preoperative diagnostic workup included physical examination, chest X-ray, lung function assessment by spirometry and arterial blood gases, liver and renal functions by laboratory tests, electrocardiography (ECG), and full assessment for anesthesia.

The postoperative follow-up evaluation was performed every 3–4 months for the first 2 years and every 6 months thereafter by CT scanning and annual endoscopy for 5 years.

Surgery

In October 2008, we introduced VATS combined with 5 cm mini-thoracotomy, a surgical technique similar to the procedure described by Osugi et al. [18] and have performed it since then in 14 patients. However, these 14 patients were excluded from the present study. The patient was placed on the right-side-up lateral position using left single lung ventilation under general anesthesia, and esophagectomy and mediastinal dissection with extensive lymphadenectomy were performed under a combination of direct visual and thoracoscopically visualized guidance through a 10 cm right thoracotomy through the fourth intercostals space. Subsequently, the patients were repositioned in the supine position, and abdominal lymphadenectomy and gastric conduit reconstruction were performed using either the open technique with an upper-middle abdominal midline laparotomy (fossa epigastrica to umbilicus) or HALS technique with 7 cm upper abdominal midline incision (through which the surgeon's left hand was inserted) and three 5–12 mm long incisions (through which the trocar tubes were inserted; Fig. 1). Following the use of different approaches to the abdominal cavity, the surgical procedures were identical between the two groups:

(1) The greater curve of the stomach was divided taking care in preservation of the gastroepiploic arcade, and the left crus was dissected and incised, allowing hiatal enlargement for lower mediastinal dissection.
(2) The gastrohepatic ligament was divided, and the right crus was dissected.
(3) The left gastric artery was transected at its base on the celiac axis, and the lymph nodes in this region and those around the proximal splenic artery and common hepatic artery were dissected en bloc.
(4) The separated anal side of the esophagus and stomach were exteriorized from the peritoneal cavity. Then the sub-total gastric conduit was constructed. Pyloroplasty was performed manually.
(5) Pull-up of the gastric conduit through the posterior mediastinal route was performed under surgeon's hand guidance, taking care in avoiding torsion, and gently delivering the stomach upward through the hiatus.
(6) Esophagogastric anastomosis was performed by circular end-to-side stapling at the neck.

Based on the location of the primary tumor and the presence or absence of metastases in the lymph nodes chain along the recurrent laryngeal nerve, patients underwent additional cervical lymph node dissection [19–21]. The insertion of a feeding tube in the jejunum was not routinely performed except for patients older than 75 years of age and those with performance status of more than 2.

We applied two energy devices to the surgical procedure involving the abdominal cavity. First, a harmonic scalpel (Ultracision; Ethicon Laboratories, Cincinnati, OH) was used in almost all steps of the dissection in HALS, while hemoclips or ligations were applied to the left gastric and left gastroepiploic vessels. Next, monopolar electrosurgery was used for cutting and coagulation and ligation to obstruct the blood vessels in open laparotomy.

Perioperative Management

In each patient, an epidural catheter was placed into the epidural space between the 5th and 6th thoracic vertebrae, for the injection of an epidural anesthetic postoperatively. Furthermore, each patient received 250 mg of methylprednisolone intravenously perioperatively. All patients were transferred to the intensive care unit (ICU) after surgery and extubated at postoperative day (POD) 1, except when requiring respiratory support. Patients who had difficulty in coughing up sputum and airway secretions underwent bronchoscopy

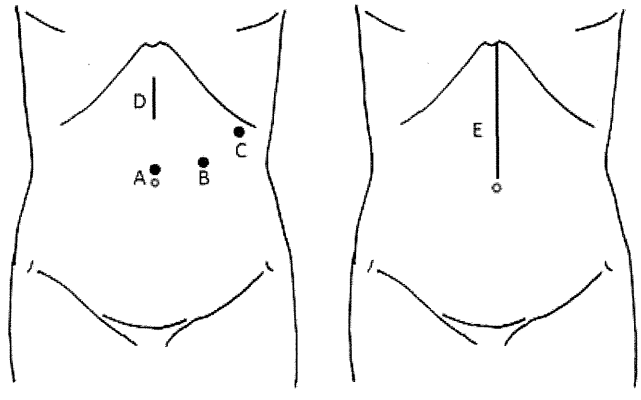

Fig. 1. Diagram of port placement and incision line for abdominal lymph node dissection and gastric mobilization in hand-assisted laparoscopic surgery (HALS) and Open laparotomy. Ports and incisions are placed in this order: Surgeon's right hand (12 mm, **A**), Camera (12 mm, **B**), Grasper (5 mm, **C**), Surgeon's left hand (7 cm, **D**), open laparotomy (**E**).

to clear these secretions from the respiratory tract. Furthermore, a mini-endotracheal tube was inserted to suction excess secretion, remove secretion difficult to discharge and/or aspirate material from the tracheobronchial tree in patients who required more than twice daily the above bronchoscopy. A nasogastric tube was inserted to suction fluids and air from the stomach. These were intermittently suctioned by the nasogastric tube which was connected to the suction device in the first 3 POD. The tube was removed at POD 7 and food intake was allowed if no evidence of leakage was clinically evident at POD 8. Enteral nutrition started at POD 2 in patients with a jejunum feeding tube. There were no differences in the perioperative management in this study.

Postoperative complications were evaluated according to the NCI-CTCAE, version 3. Grade 2 and over adverse events were defined as the appearance of complications. Systemic inflammatory response syndrome (SIRS) was defined by the presence of at least two of the following criteria: (1) heart rate >90 beats/min, (2) respiratory rate >20 breaths/min, (3) body temperature >38 or <36°C, and (4) blood leukocyte count $>12 \times 10^3/mm^3$ or $<4 \times 10^3/mm^3$.

Statistical Analysis

Values were expressed as mean ± standard deviation. Comparison of data of two groups was undertaken using the chi-squared test for categorical data, and the Student *t*-test or Mann–Whitney *U*-test for continuous data. Survival was calculated with the Kaplan–Meier method and differences between groups were evaluated by the log-rank test. Statistical significance for each model was set at $P < 0.05$.

RESULTS

Patients' Demographics

Table I summarizes the characteristics of each group. There was a greater utilization of neoadjuvant chemotherapy in the HALS group ($P = 0.02$), and a greater proportion of cases with advanced disease, albeit insignificantly, in HALS group than Open group ($P = 0.08$). Thus, the differences between the two groups reflect the histopathological changes in the treatment of resectable esophageal cancers in our institution. There was no difference in age, sex, performance status, body mass index (BMI), and preoperative comorbidity between the two groups.

TABLE I. Patient Characteristics and Pathology Demographics of Patients of the Hand-Assisted Laparoscopic Surgery (HALS) and Open Surgery (Open) Groups

	HALS	Open	*P*-value
Number	109	107	
Procedure period	Oct 2007–Jan 2010	Jan 2005–Sep 2007	
Age (years)[a]	64.6 ± 8.5	64.7 ± 8.0	0.99
Sex (M:F)	87:22	95:12	0.1
Performance status (0:1/2)	88:20/1	84:23/0	0.74
BMI (kg/m^2)[a]	21.1 ± 3.1	21.0 ± 2.8	0.62
Location (Ut:Mt:Lt)	23:59:27	18:49:40	0.25
cT (1:2:3:4)	11:24:57:17	17:26:47:17	0.5
cN (0:1)	35:74	43:64	0.22
cStage (1:2:3:4)	7:34:48:20	13:37:44:13	0.08
Neoadjuvant therapy (none:CT:CRT)	24:69:16	39:48:20	0.02
Comorbidity			
Cardiovascular disease	6	4	0.54
Diabetes	10	6	0.31
Hypertension	14	16	0.65
Pulmonary diseases	11	13	0.63
Liver dysfunction	5	9	0.25
Other cancers	5	2	0.25

BMI, body mass index; Ut, upper third of the esophagus; Mt, middle third; Lt, lower third; CT, chemotherapy; CRT, chemoradiotherapy.
[a]Data are mean or number of patients.

Operative Outcome

The operative outcome is summarized for each group in Table II. The mean operating time and time of abdominal manipulation were similar in the two groups. The total and abdominal blood loss was significantly less in the HALS group than in the Open group. The amount of blood transfusion was significantly less in the HALS group than in the Open group (1.2 U vs. 2.1 U; $P = 0.008$). On the other hand, intraoperative fluid balance was similar in the two groups (HALS: 2.2 L, Open: 2.3 L; $P = 0.21$). Three-field lymphadenectomy was performed in 56 and 60 patients in the Open and HALS groups, respectively ($P = 0.69$). None of the HALS procedure required conversion to open surgery.

Postoperative Outcome

Table III summarizes the postoperative outcome of both groups. Serum C-reactive protein (CRP) levels at POD 1, 3, and 7 were significantly lower in the HALS group than Open group. The duration of SIRS was also significantly shorter in the HALS group than in the Open group (2.3 days vs. 3.5 days; $P = 0.0002$). The durations of ICU and hospital stay were similar in both groups. The volume of discharge from the abdominal drain on POD 2 and POD 3 was significantly lower in the HALS group than Open group. The time until abdominal drain removal was similar in both groups. The volume of discharge from the nasogastric tube on POD 1, 2, and 3 was also significantly lower in the HALS group than in the Open group (data not shown). The average volume of discharge from the chest drain in the 5 days was similar in both groups (HALS: 221 ml, Open: 234 ml; $P = 0.11$). The time to passing the first flatus was significantly shorter in the HALS group than Open group. The duration of analgesia was similar in both groups (data not shown).

The postoperative complications are shown in Table III. Complications occurred in 64 of 216 patients (29.6%), and the rate in the HALS group (23.9%) was lower than the Open group. The rate of pneumonia in the HALS group was lower, albeit statistically

TABLE II. Operative Outcome in the Hand-Assisted Laparoscopic Surgery (HALS) and Open Surgery (Open) Groups

	HALS	Open	*P*-value
Cervical LN dissection			
No	49	51	0.69
Yes	60	56	
Number of resected abdominal LN	19.3 ± 7.1	20.8 ± 8.3	0.14
Total operating time (min)	452 ± 65	456 ± 69	0.67
Abdominal operating time (min)	172 ± 49	172 ± 44	0.98
Total blood loss (ml)	695 ± 369	1101 ± 540	0.0001
Blood loss in abdominal procedure (ml)	233 ± 222	591 ± 400	0.0001
Amount of transfusion (unit)	1.2 ± 2.1	2.1 ± 2.6	0.008
Intraoperative fluid balance (L)	2.2 ± 0.88	2.3 ± 0.77	0.21
Type of resection			
R0	108	105	0.55
R1	1	2	

LN, lymph node.
Data are mean ± SD or number of patients.

TABLE III. Postoperative Outcome of the Hand-Assisted Laparoscopic Surgery (HALS) and Open Surgery (Open) Groups

	HALS (%)	Open (%)	*P*-value
Complications	26 (23.9)	38 (35.5)	0.11
Anastomotic leakage	6 (5.5)	4 (3.7)	0.54
Pneumonia	7 (6.4)	15 (14.0)	0.062
Chylothorax	2 (1.8)	2 (1.9)	0.98
Hemorrhage	1 (0.9)	3 (2.8)	0.30
Vocal cord palsy	17 (15.6)	20 (18.7)	0.55
Arrhythmias	3 (2.8)	6 (5.6)	0.29
Delirium	0 (0)	2 (1.9)	0.15
Complete conduit ischemia	0 (0)	2 (1.9)	0.15
Mini-endotracheal tubing	21 (19.3)	52 (48.6)	<0.0001
Duration of oxygen use (days)	5.2 ± 6.7	7.8 ± 10.2	0.026
Hospital stay (days)	29.9 ± 15	33.0 ± 25	0.28
In-hospital deaths	0 (0)	2 (1.9)	0.24
Duration of SIRS (days)	2.32 ± 1.6	3.50 ± 2.7	0.0002
Postoperative CRP (mg/dl)			
POD 1	6.3 ± 1.9	7.6 ± 2.7	0.0001
POD 3	13.6 ± 6.4	15.5 ± 5.6	0.019
POD 7	5.4 ± 4.8	6.6 ± 4.1	0.046
Time to passing flatus (days)	3.8 ± 1.9	4.8 ± 1.8	0.0001
Discharge from abdominal drain (ml)			
POD 1	148 ± 134	165 ± 206	0.48
POD 2	110 ± 99	172 ± 186	0.0032
POD 3	77.7 ± 126	142 ± 231	0.027
Discharge from chest drain (ml)	221 ± 43	234 ± 69	0.11

SIRS, systematic inflammatory response syndrome; CRP, C-reactive protein; POD, postoperative day.
Data are mean ± SD or number (%) of patients.

insignificant, than in the Open group (6.4% vs. 14.0%; $P = 0.06$). The proportion of patients who required bronchoscopy was significantly lower in the HALS group (27.5%) than the Open group (60.8%; $P < 0.0001$). The proportion of patients who required insertion of a mini-endotracheal tube was significantly lower in the HALS group (19.3%) than the Open group (48.6%; $P < 0.0001$). The duration of oxygen use during the postoperative period was significantly shorter in the HALS group (5.2 days) than in the Open group (7.8 days; $P = 0.026$). Vocal cord palsy occurred in 37 of 216 patients (17.1%), but was only transient, and the rate of affected patients was similar in both groups.

Outcome of Cancer Treatment

The number of harvested abdominal lymph nodes was not different between the two groups (mean: Open = 20.8, HALS = 19.3; $P = 0.14$). The numbers of patients who developed recurrence of abdominal lymph nodes after surgical dissection were 4 and 2 patients in the Open and HALS group, respectively. The median follow-up period was 35.4 months in the Open group and 20.1 months in the HALS group. The disease-free survival rates of the two groups are shown in Figure 2. The 2-year disease-free survival rate of the Open group was 53.0% compared with 64.9% for the HALS group, but the difference was not statistically significant ($P = 0.08$).

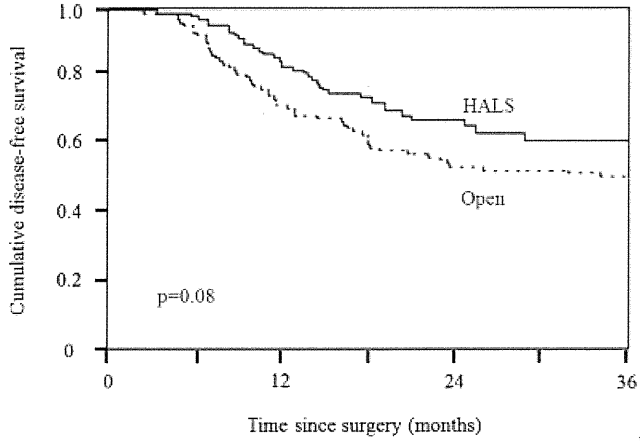

Fig. 2. Kaplan–Meier curves of disease-free survival rates of patients of the HALS group and Open group.

DISCUSSION

The main finding of the present study was the superiority of HALS compared with open laparotomy, based on the following results: (1) HALS resulted in a significantly lower blood loss, (2) HALS did not require longer operating time, (3) low rate of perioperative complications after HALS, (4) significant reduction of postoperative CRP level after HALS, and (5) HALS was associated with early recovery from SIRS. These results indicate that HALS is less invasive than Open laparotomy.

The study showed a lower rate of postoperative complications, especially pulmonary complications, after HALS than open surgery. In addition, it showed a significantly lower need for bronchoscopy and use of a mini-endotracheal tube, and shorter use of oxygen. This could be due to the intact abdominal wall with no associated pain during coughing. This result is clinically meaningful since pulmonary complications were one of the most common reasons of postoperative mortality.

The present study also showed a lower incidence of discharge from the nasogastric tube and abdominal drain and shorter time to the passage of the first flatus after HALS than Open laparotomy. These findings suggest that HALS allows earlier restoration of digestive function, in agreement with previous studies that reported decreased gut dysmotility after endoscopy-assisted laparoscopic surgery and HALS in patients with various colorectal and gastric conditions [22,23].

Admittedly, one must take account of the effects of the ancillary surgical instrumentations, especially energy devices, on the outcome. In the present study, the energy source used in HALS procedure was ultrasonic-activated shears. In comparison, open procedure was performed using monopolar electrosurgery. Some studies reported that the use of the new energy devices such as ultrasonic-activated shears is associated with lower blood loss compared with conventional hemostatic techniques such as monopolar electosurgery [24,25]. Whereas the use of the energy device in HALS could have contributed to the lower blood loss, relative to the open procedure, a larger proportion of the low blood loss in HALS was due to the procedure itself; HALS allowed the surgeon to identify and treat the fine anatomic structures through better visualization using the endoscope, and required the use of only a small abdominal incision.

Another important issue that needs to be discussed is the potential bias introduced by the selection of the surgical operation. In present study, the following aspects of the study design should minimize any such bias. The operative procedure was uniformly performed in all patients because three or all surgeons of four upper-GI specialists participated in each operation as the chief surgeon or assistant surgeon during the entire period of this study. Analysis of the differences among surgeons showed similar operative and postoperative

outcomes among the four surgeons (data not shown). There were also no differences in the operative and postoperative outcomes according to the time these procedures were conducted (early and late periods) between open laparotomy and HALS (data not shown).

We employed HALS, but not laparoscopic surgery, as minimally invasive esophagectomy. Many studies have reported that laparoscopic surgery has several advantages based on the minimal access approach such as lower operative blood loss, less pain, earlier recovery of bowel activity, and a shorter hospital stay [22,26,27]. However, the laparoscopic procedures are time-consuming and technically demanding and hence have a long learning curve [28]. In comparison, HALS permits direct tactile sensation and allows gentle retraction of large masses of tissues, which enhance exposure, identification of anatomic structures, rapid control of bleeding, and avoidance of tissue injures [14–16]. In particular, HALS was safer and more useful than laparoscopic surgery in the reservation and management of gastric conduit reconstruction and abdominal lymphadenectomy during radical esophagectomy, because it allowed the avoidance of gastric injury commonly seen following grasp with forceps, and assist in pulling up the gastric conduit under surgeon's hand guidance without any torsion. We have experienced only one complete conduit ischemia during laparoscopic surgery but none during HALS. Furthermore, mastering the HALS procedure hardly required a transitional period from open procedure because it is easy to learn and easy to teach as reported previously [29]. The widespread use of laparoscopic surgery, which has been performed in 270 patients with gastric cancer in our institution since 2007, attest to the ease of learning and executing this procedure. Therefore, we consider HALS as a more user-friendly and practical alternative to laparoscopic surgery for gastric conduit reconstruction and abdominal lymphadenectomy in radical esophagectomy.

Our concerns were the oncological results of HALS procedure in esophagectomy. In this study, the number of lymph nodes harvested and the rate of complete pathological resection were not different between the two procedures. Several studies have shown that the total number of surgically removed lymph nodes was independently associated with survival in esophageal cancer [30,31]. In fact, the number of harvested lymph nodes is considered a useful surrogate marker for surgical curability.

Analysis of the survival rate is the most important outcome in assessment of oncological results. In this cohort, there was no difference in the recurrence-free survival between the two groups, and the survival rate was comparable to that reported in other published series [32,33,11]. While our results showed the clear benefits of HALS over conventional open surgery for abdominal lymphadenectomy and gastric conduit reconstruction in radical esophagectomy, further assessment of prognosis and recurrence over a longer period is needed to provide meaningful long-term follow-up data.

CONCLUSIONS

The HALS procedure seems potentially feasible and beneficial for patients with esophageal cancer since it would reduce operative blood loss, the incidence of postoperative pneumonia and systemic inflammation, and allows early restoration of digestive function while retaining equally oncological curative effect compared with the open procedure.

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A phase I study of vaccination with NY-ESO-1f peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen

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We conducted a phase I clinical trial of a cancer vaccine using a 20-mer NY-ESO-1f peptide (NY-ESO-1 91–110) that includes multiple epitopes recognized by antibodies, and CD4 and CD8 T cells. Ten patients were immunized with 600 μg of NY-ESO-1f peptide mixed with 0.2 KE Picibanil OK-432 and 1.25 ml Montanide ISA-51. Primary end points of the study were safety and immune response. Subcutaneous injection of the NY-ESO-1f peptide vaccine was well tolerated. Vaccine-related adverse events observed were fever (Grade 1), injection-site reaction (Grade 1 or 2) and induration (Grade 2). Vaccination with the NY-ESO-1f peptide resulted in an increase or induction of NY-ESO-1 antibody responses in nine of ten patients. The sera reacted with recombinant NY-ESO-1 whole protein as well as the NY-ESO-1f peptide. An increase in CD4 and CD8 T cell responses was observed in nine of ten patients. Vaccine-induced CD4 and CD8 T cells responded to NY-ESO-1 91–108 in all patients with various HLA types with a less frequent response to neighboring peptides. The findings indicate that the 20-mer NY-ESO-1f peptide includes multiple epitopes recognized by CD4 and CD8 T cells with distinct specificity. Of ten patients, two with lung cancer and one with esophageal cancer showed stable disease. Our study shows that the NY-ESO-1f peptide vaccine was well tolerated and elicited humoral, CD4 and CD8 T cell responses in immunized patients.

Key words: NY-ESO-1, cancer vaccine, long peptide, immune response
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The NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum.[1,2] NY-ESO-1 expression is observed in a wide range of human malignancies,[3,4] but the expression is restricted to germ cells in the testes in normal adult tissues.[1,3] Therefore, NY-ESO-1 has emerged as a prototype of a class of cancer/testis (CT) antigens.[5]

More than 100 patients with NY-ESO-1-expressing tumors have received the NY-ESO-1 vaccine either as full-length recombinant protein given as protein alone, with ISCOMATRIX® or cholesterol-bearing hydrophobized pullulan (CHP), delivered in a recombinant vaccinia or fowlpox vector, or as the NY-ESO-1b peptide given with various adjuvants.[6–11] These studies established safety with various preparations of the NY-ESO-1 vaccine, showing toxicity to be limited to Grade 1 or 2 injection-site reactions or flu-like

symptoms, *e.g.*, fever and malaise. Vaccination with these preparations has been shown to enhance or generate NY-ESO-1 immune responses in the majority of patients by immune monitoring using sera and peripheral blood lymphocytes.

CHP is a newly developed antigen delivery vehicle that can be used to formulate nanoparticles, including protein antigens.[12,13] Both CD4 and CD8 T cells are efficiently activated by DCs pulsed with a complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) *in vitro*.[14] In a phase I clinical trial, we immunized nine cancer patients with CHP-NY-ESO-1 and showed that the vaccine had potent capacity to induce the NY-ESO-1 antibody in all of nine vaccinated patients.[15] The regions in the NY-ESO-1 molecule recognized by antibodies from vaccinated patients were similar to those recognized by antibodies in nonvaccinated cancer patients with spontaneous immunity. Especially, we showed that NY-ESO-1 91–108 was recognized in six of nine vaccinated patients and in eight of nine nonvaccinated, seropositive patients.[15] This region was defined as the most dominant serological antigenic epitope. A CHP-NY-ESO-1 vaccine also elicited CD4 and CD8 T cell responses in immunized patients.[16] An increase in the CD4 and CD8 T cell responses was observed in all of two initially seropositive and five of seven initially seronegative patients after vaccination. Analysis of T cell responses against overlapping peptides spanning the NY-ESO-1 molecule revealed that two dominant NY-ESO-1 regions, regions II (73–114) and III (121–144), were recognized by CD4 and CD8 T cells in most patients irrespective of their HLA type. Importantly, the most dominant peptide region (91–108) eliciting an antibody response was also included in region II. Essentially similar findings were obtained by studies using other preparations of NY-ESO-1 protein vaccine.[9,11]

Protein vaccines containing multiple epitopes appear to be promising in eliciting strong immune responses, but there are several constraints against their general use. To produce sufficient amounts of recombinant protein for a vaccine, a huge fermentation facility is necessary. Operating such facilities at GMP grade is extremely costly. Furthermore, there are several technical difficulties to be overcome to obtain highly purified protein at a sufficient yield such as removing bacterial or other contaminants from the preparation.

CD8 and CD4 T cells induced by immunization with NY-ESO-1 class I and II short epitope peptides, respectively, have been shown to be of low affinity and do not recognize naturally processed NY-ESO-1.[17] However, it has recently been shown that a long peptide is capable of inducing antibody, CD4 and CD8 T cell responses *in vivo* as the protein antigen.[18,19]

On the basis of these findings, in our study, we investigated the immunogenicity of a long peptide spanning a peptide region NY-ESO-1 91–110 for use as a vaccine. We examined the safety of repeated vaccinations with NY-ESO-1f peptide at a dose of 600 μg mixed with immune adjuvants Picibanil® OK-432 and Montanide® ISA-51. Furthermore, we monitored the humoral, CD4 and CD8 T cell responses in patients receiving NY-ESO-1f peptide vaccine and recorded tumor responses.

Material and Methods

NY-ESO-1f peptide vaccine

NY-ESO-1f peptide (NY-ESO-1 91–110: YLAMPFATPMEAELARRSLA) was manufactured by CLINALFA, Merck Biosciences (Läufelfingen, Switzerland) and provided by the Ludwig Institute for Cancer Research, New York. The vaccine, consisting of 600 μg of NY-ESO-1f peptide, 0.2KE OK-432 (Picibanil™; Chugai Pharmaceutical, Tokyo, Japan) and 1.25 ml ISA-51(Montanide™; Seppic, Paris, France), was emulsified under sterile conditions. All synthesis, production, formulation and packaging of the investigational agent were in accordance with applicable current Good Manufacturing Practices and met the applicable criteria for use in humans.

Study design

A phase I clinical trial of the NY-ESO-1f peptide vaccine was designed to evaluate the safety, immune response and tumor response. Patients with advanced cancers that were refractory to standard therapy and expressed NY-ESO-1 as assessed by immunohistochemistry (IHC) were eligible. Cancer patients including six patients with esophageal cancer, three patients with non-small-cell lung cancer and one patient with gastric cancer were enrolled in a washout period of at least 4 weeks after surgery, chemotherapy or radiation therapy. The vaccines were administered subcutaneously once every 3 weeks in six doses. Four weeks after the last administration, the safety, immune response and tumor response were evaluated. Thereafter, the vaccine was administered additionally. The ten patients received 5–21 immunizations.

The protocol was approved by the Ethics Committee of Osaka, Tokyo and Okayama Universities in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling in the study. The study was conducted in compliance with Good Clinical Practice. The study was registered in the University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial number: UMIN000001260) on July 24, 2008 (UMIN-CTRURL: http://www.umin.ac.jp/ctr/index.htm).

Blood samples

Peripheral blood was drawn from the patients before vaccination, at each time point of immunization and 4 weeks after the last immunization. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated by density gradient centrifugation using lymphoprep (Axis Shield PoC AS, Oslo, Norway). A CD8 T cell-enriched population was obtained from PBMCs using CD8 microbeads with a large-scale column and a magnetic device (Miltenyi Biotec, Auburn, CA). A CD4 T cell-enriched population was then obtained from the residual cells using CD4 microbeads. The final residual cells were used as a

CD4- and CD8-depleted population. These populations were stored in liquid N_2 until use. HLA typing of PBMCs was done by sequence-specific oligonucleotide probing and sequence-specific priming of genomic DNA using standard procedures.

Overlapping peptides

The following series of 28 overlapping NY-ESO-1 18-mer peptides spanning the protein were used: 1–18, 7–24, 13–30, 19–36, 25–42, 31–48, 37–54, 43–60, 49–66, 55–72, 61–78, 67–84, 73–90, 79–96, 85–102, 91–108, 97–114, 103–120, 109–126, 115–132, 121–138, 127–144, 133–150, 139–156, 145–162, 149–166, 153–170 and 156–173. A 30-mer peptide, 151–180, was also used. These peptides were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl chemistry on a Multiple Peptide Synthesizer (AMS422; ABIMED, Langenfeld, Germany) at Okayama University.

ELISA

Recombinant NY-ESO-1 protein was prepared as described previously.[1] Recombinant NY-ESO-1 protein (1 μg/ml) or NY-ESO-1f peptide (10 μg/ml) in a coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) was adsorbed onto 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with PBS and blocked with 200 microliters per well of 5% FCS/PBS for 1 hr at room temperature. Then, 100 μl of serially diluted serum was added to each well, and it was incubated for 2 hr at room temperature. After extensive washing, horseradish peroxidase-conjugated goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells, and the plates were incubated for 1 hr at room temperature. After washing and development, absorbance at 490 nm was read. Recombinant murine Akt protein[20] and ovalbumin (OVA, albumin from chicken egg white; Sigma, St. Louis, MO) were used as control proteins.

In vitro stimulation of CD4 and CD8 T cells

Frozen cells were thawed and resuspended in AIM-V (Invitrogen, Carlsbad, CA) medium supplemented with 5% heat-inactivated pooled human serum (CM) and kept at room temperature for 2 hr. CD4- and CD8-enriched populations (2×10^6) were cultured with irradiated (30 Gy), autologous CD4- and CD8-depleted PBMCs (2×10^6) in the presence of the 28 18-mer overlapping peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (1 μg/ml for each peptide) in 2 ml of CM supplemented with 10 U/ml rIL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/ml rIL-7 (Peprotech, London, UK) in a 24-well culture plate at 37°C in a 5% CO_2 atmosphere for 12 days. For the second stimulation, 1×10^6 instead of 2×10^6 responder cells were used in the culture described above.

IFNγ capture assay

The IFNγ capture assay[21,22] was carried out according to the manufacturer's protocol (Miltenyi Biotec). Briefly, 2×10^5 responder CD4 and CD8 T cells were stimulated for 4 hr at 37°C in a 5% CO_2 atmosphere with paraformaldehyde (PFA, 0.2%)-treated autologous CD4- and CD8-depleted PBMCs (2×10^5) prepulsed with the peptides. The cells were then washed and suspended in 100 μl of cold RPMI medium and treated with bispecific CD45 and IFNγ mouse antibodies (IFNγ catch reagent) (2 μl) for 5 min on ice. The cells were then diluted in AIM-V medium (1 ml) and placed on a slow rotating device (Miltenyi Biotec) to allow IFNγ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 45 min, the cells were washed with cold buffer and treated with 7AAD (7-amino-actinomycin D, Becton Dickinson, Mountain View, CA), PE-conjugated anti-IFNγ (detection reagent) and FITC-conjugated anti-CD4 or CD8 mAbs for staining. After incubation for 10 min at 4°C, the cells were washed and analyzed with a FACS Calibur (Becton Dickinson). Dead cells were sorted by 7AAD staining. The data were analyzed with FlowJo software (Tree Star, Ashland, OR). A net population of IFNγ-captured CD4 and CD8 T cells of more than 0.1% was considered significant.

Immunohistochemistry

IHC was performed as described previously.[3] E978[23] and EMR8-5 (Funakoshi, Tokyo, Japan)[24] mAbs were used to analyze NY-ESO-1 and HLA class I expression, respectively. The reaction was evaluated as +++ (>50% stained cells), ++ (25–50% stained cells), + (5–25% stained cells) and − (<5% stained cells).

Results

Patient characteristics

Table 1 shows a list of the ten patients enrolled in the study. They included six patients with esophageal cancer, three with non-small-cell lung cancer and one with gastric cancer who were refractory to the standard therapy. Expression of NY-ESO-1 and MHC class I in the tumor was confirmed in biopsy or surgical specimens by IHC in all patients upon entry into the study. Nine patients completed the study with six injections of the NY-ESO-1f peptide with Picibanil and Montanide, but patient OS-f01 was withdrawn from the study after five doses of the vaccine because of disease progression. All patients were considered evaluable for toxicity, immunological and clinical responses. Six patients with a prolonged disease course were allowed to continue vaccination after a cycle of six doses of the vaccine.

Toxicity

Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events v.3.0.[25] As shown in Table 1, six patients showed Grade 1 fever (38–39°C) that subsided within a few days without any medication. All patients except OY-f04 developed an injection-site reaction (Grade 1 or 2). TK-f01, TK-f04 and TK-f05 developed a Grade 2 injection-site reaction early after the first vaccination. The reaction appeared 48–72 hr after

Table 1. Patient characteristics

ID	Age/Sex	Cancer/Histology	Vaccination	Vaccine-related toxicity
OY-f04	59/M	Esophageal cancer Squamous cell carcinoma	6	Fever (Grade 1)
OS-f01	66/M	Esophageal cancer Squamous cell carcinoma	5	Fever (Grade 1), injection-site reaction (Grade 1)
OS-f03	61/M	Gastric cancer Adenocarcinoma	26	Fever (Grade 1), injection-site reaction (Grade 1), induration (Grade 1)
OS-f06	51/M	Esophageal cancer Squamous cell carcinoma	6	Injection-site reaction (Grade 1), induration (Grade 1)
OS-f08	69/M	Esophageal cancer Squamous cell carcinoma	13	Injection-site reaction (Grade 1), induration (Grade 1)
TK-f01	59/M	Lung cancer Adenocarcinoma	12	Fever (Grade 1), injection-site reaction (Grade 2), induration (Grade 2)
TK-f02	67/M	Lung cancer Adenocarcinoma	12	Fever (Grade 1), injection-site reaction (Grade 2), induration (Grade 2)
TK-f03	72/M	Esophageal cancer Squamous cell carcinoma	7	Injection-site reaction (Grade 1)
TK-f04	37/F	Lung cancer Adenocarcinoma	6	Fever (Grade 1), injection-site reaction (Grade 2), induration (Grade 2)
TK-f05	71/M	Esophageal cancer Squamous cell carcinoma	11	Injection-site reaction (Grade 2), induration (Grade 2)

injection, and erythema was accompanied by swelling. Grade 2 induration occurred thereafter without retraction. In patient TK-f02, erythema was first observed after the third injection and accompanied induration after the fifth injection (Supporting Information Fig. 1). The induration gradually subsided during the course of the treatment. No augmentation of the reaction intensity was observed at previous injection sites. No severe adverse events related to the drug were observed.

Antibody response to the NY-ESO-1 whole protein and NY-ESO-1f peptide

The NY-ESO-1 antibody response in the patients vaccinated with NY-ESO-1f peptide with Picibanil and Montanide was evaluated by ELISA using recombinant NY-ESO-1 protein and the NY-ESO-1f peptide. Figure 1 shows the results of ELISA with sera from each patient obtained at the baseline and after each vaccination. The patients include two baseline seropositive patients (OS-f03 and TK-f03) and eight baseline seronegative patients. The sera from two seropositive patients also reacted to the NY-ESO-1f peptide, consistent with our previous observation that the NY-ESO-1f peptide represents an immunodominant B cell epitope.[15]

In the seropositive patients, an increase in the NY-ESO-1 antibody response was observed after vaccination. In seven of eight baseline seronegative patients, the NY-ESO-1 antibody response was induced after three to six vaccinations and increased gradually thereafter. The response against NY-ESO-1 protein could be detected in higher dilutions of sera than that against the NY-ESO-1f peptide. The kinetics of the responses against NY-ESO-1 protein and NY-ESO-1f peptide were basically the same.

CD4 and CD8 T cell responses in patients after NY-ESO-1f peptide vaccination

CD4 and CD8 T cell responses were evaluated in the ten patients by the IFNγ capture assay. Patient HLA genotypes are listed in Table 2. CD4 and CD8 T cell-enriched populations were cultured for 12 days with irradiated autologous CD4- and CD8-depleted PBMC in the presence of a mixture of 28 overlapping 18-mer peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (1°IVS). The cells from the stimulation culture were then assayed for IFNγ secretion by stimulating them for 4 hr with PFA-treated CD4- and CD8-depleted PBMC prepulsed with the peptide. To confirm the response, the cells were also analyzed after secondary *in vitro* stimulation (2°IVS). Figure 2 shows the representative FACS plot results from the two patients for three different time points before and after vaccination in 1° and 2°IVS. The net percentage of IFNγ-secreting cells of the total number of CD4 and CD8 T cells in cultures was determined. Values >0.1% were considered significant. As shown in Figure 3 and Table 3, a CD4 T cell response was detected in nine of ten patients in 1°IVS. In seropositive patient

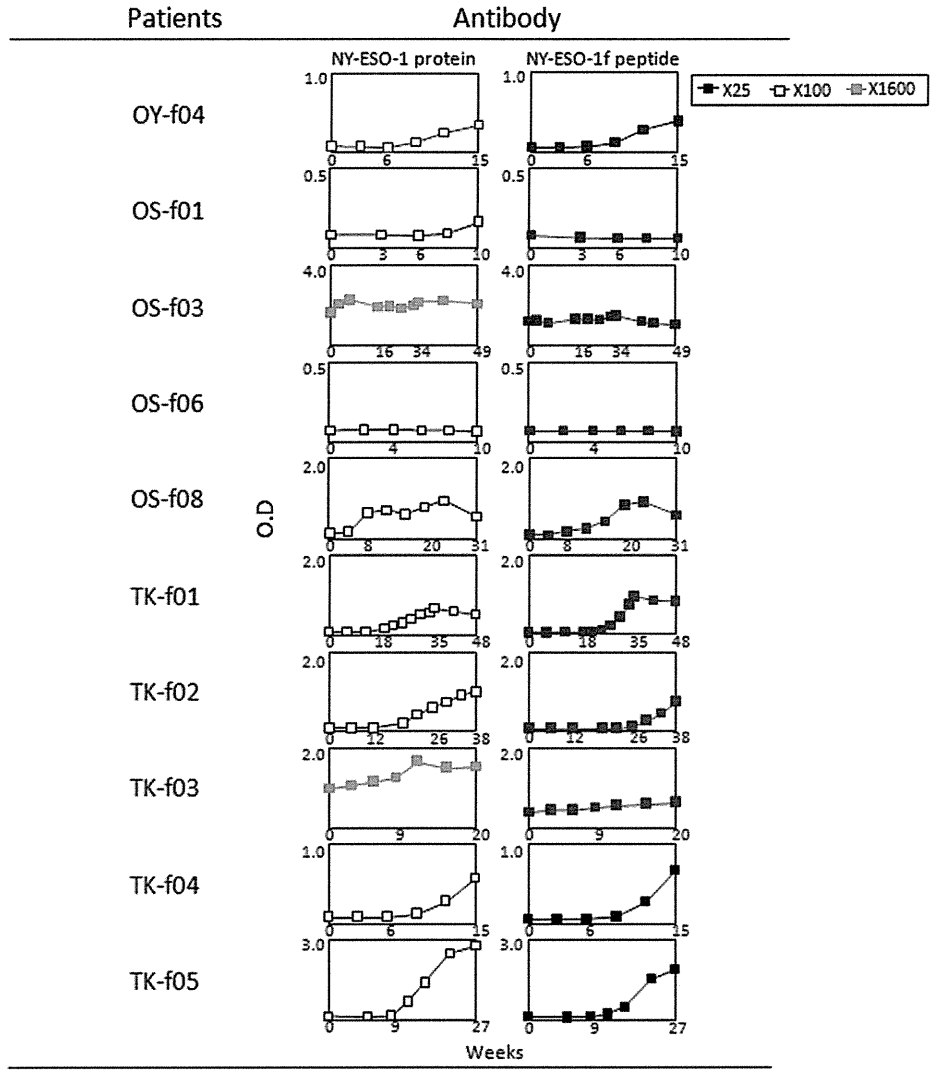

Figure 1. Antibody response to the NY-ESO-1 protein or NY-ESO-1f peptide. Sera obtained at the baseline and after each vaccination were used for ELISA. The O.D. values (490 nm) for the NY-ESO-1f peptide at a serum dilution of 1:25 (closed) and for NY-ESO-1 protein at a serum dilution of 1:100 (open) for seronegative patients or 1:1,600 (gray) for seropositive patients are shown. The O.D. values of the control protein (Akt) were less than 0.05. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TK-f03, a strong CD4 T cell response was observed before vaccination and increased after vaccination. In another seropositive patient, OS-f03, and a seronegative patient, OS-f01, a strong CD4 T cell response was observed after vaccination. In the remaining six seronegative patients, a moderate CD4 T cell response was induced after vaccination. The frequency of IFNγ-producing CD4 T cells increased and reached a plateau after repeated vaccinations in all patients except OS-f01 and OS-f08. In OS-f01, the response could be examined only with the cells taken after the first and third vaccinations. In OS-f08, the response was transient. In patient OS-f06, the CD4 T cell response was barely detectable.

As shown in Figure 3 and Table 3, a CD8 T cell response was also detected in nine of ten patients in 1°IVS. In seropositive patient TK-f03, IFNγ-producing CD8 T cells were detected before vaccination and their frequency increased after vaccination. In another seropositive patient, OS-f03, and seronegative patients TK-f01, TK-f02 and TK-f04, a robust and sustained CD8 T cell response was induced after vaccination. Even a single vaccination elicited a response in these patients. In patient OS-f08, an increase in CD8 T cell response was observed after the seventh vaccination. In patients OY-f04 and TK-f05, the CD8 T cell response was transient. No CD8 T cell response was detected in patient OS-f01.

Determination of NY-ESO-1 peptides recognized by CD4 and CD8 T cells in patients vaccinated with NY-ESO-1f peptide with Picibanil and Montanide

CD4 and CD8 T cell responses for individual overlapping peptides were analyzed by an IFNγ capture assay. As shown

Table 2. Patient HLA

ID	A	C	B	DR	DQ	DP
OY-f04	*2402, –	*0702, –	*0702,*4001	*0101,*0901	*0303,*0501	*0201,*0402
OS-f01	*0201,*1101	*0304,*0401	*1301,*1501	*0406,*1202	*0301,*0302	*0201,*0501
OS-f03	*1101,*2402	*0401,*0801	*1501,*4006	*0406,*0901	*0302,*0303	*0201,*0501
OS-f06	*0201,*2402	*0102, –	*5401,*5901	*0405,*0803	*0401,*0601	*0201, –
OS-f08	*1101,*2402	*0303,*1202	*3501,*5201	*0403,*1502	*0302,*0601	*0201,*0901
TK-f01	*2402, –	*0303,*1202	*3501,*5201	*0405,*1502	*0401,*0601	*0201,*0901
TK-f02	*2402,*3101	*1202,*1402	*5101,*5201	*0405,*1502	*0401,*0601	*0301,*0901
TK-f03	*0201,*2402	*0303,*0401	*1501,*4002	*0406,*0901	*0302,*0303	*0201,*0501
TK-f04	*0201,*1101	*0303,*0801	*3501,*4801	*0406,*0802	*0302, –	*0201,*0501
TK-f05	*0206, –	*0304,*0702	*3902,*4002	*0405,*0901	*0303,*0401	*0301,*1301

HLA-A, C, B, DR, DQ and DP genotypes were determined by high-resolution molecular typing using PBMC from patients.

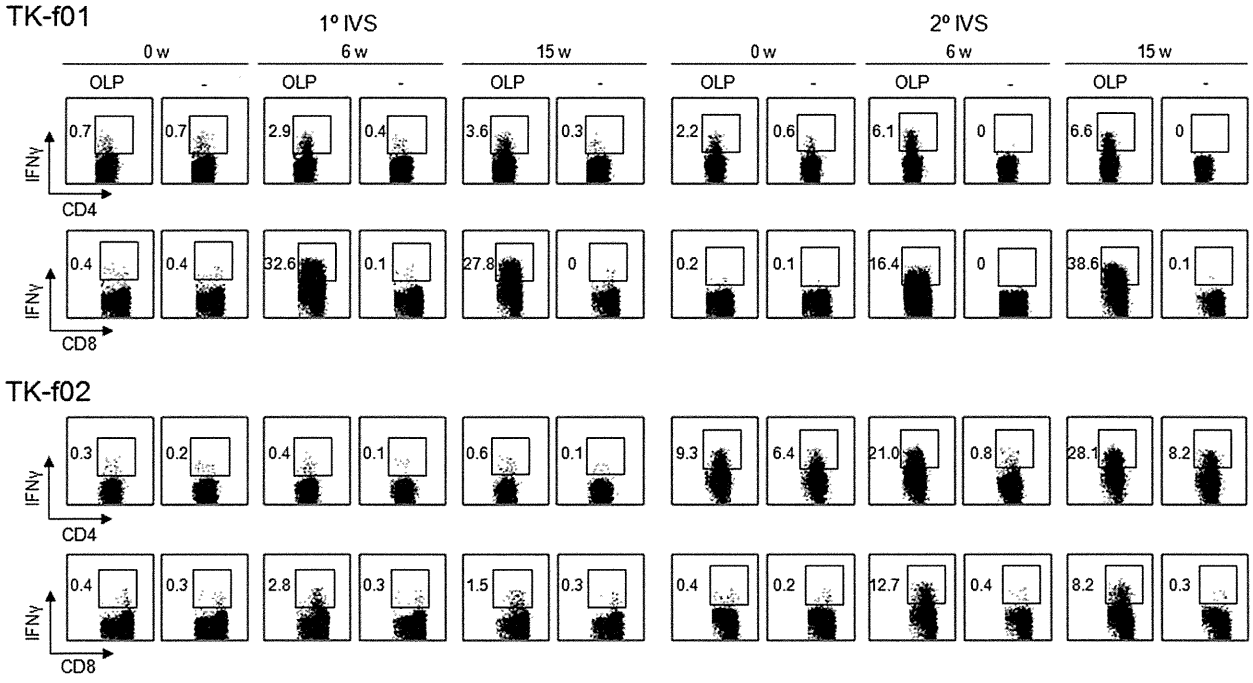

Figure 2. IFNγ capture assay of CD4 and CD8 T cells. MACS beads-purified CD4 and CD8 T cells (2×10^6) obtained from PBMC of vaccinated patients at three time points were stimulated once (1°IVS) for 12 days or twice (2°IVS) for 24 days with irradiated autologous CD4- and CD8-depleted PBMC (2×10^6) in the presence of a mixture of 28 18-mer overlapping peptides and a 30-mer C-terminal peptide spanning the entire NY-ESO-1 protein (1 μg/ml for each peptide). The cells (2×10^5) from the stimulation culture were assayed for IFNγ secretion by stimulating them for 4 hr with PFA-treated CD4- and CD8-depleted PBMC (2×10^5) prepulsed or not prepulsed with a mixture of the peptides (OLP) using FACS. The net percentage of IFNγ-secreting cells of the total number of CD4 and CD8 T cells in cultures was determined. Values >0.1% were considered significant.

in Supporting Information Figure 2, the NY-ESO-1f peptide vaccine-induced CD4 T cells showed a response to peptide 16 (NY-ESO-1 91-108) in all six patients analyzed and to peptide 15 (NY-ESO-1 85-102) or 17 (NY-ESO-1 97-114) in two patients, respectively. Similarly, vaccine-induced CD8 T cells showed a response to peptide 16 in all six patients analyzed and to peptide 15 (NY-ESO-1 85-102) in two patients. These patients showed different HLA types (Table 2). The results indicated that the 20-mer NY-ESO-1f peptide includes multiple HLA class II and class I binding epitopes recognized by CD4 and CD8 T cells, respectively, with distinct specificity (manuscript in preparation). No recognition of other peptides was observed except for CD4 T cells from OS-f08, which showed a moderate response to peptides 2 and 20 and rather lower responses to other multiple peptides, probably because of the high background.

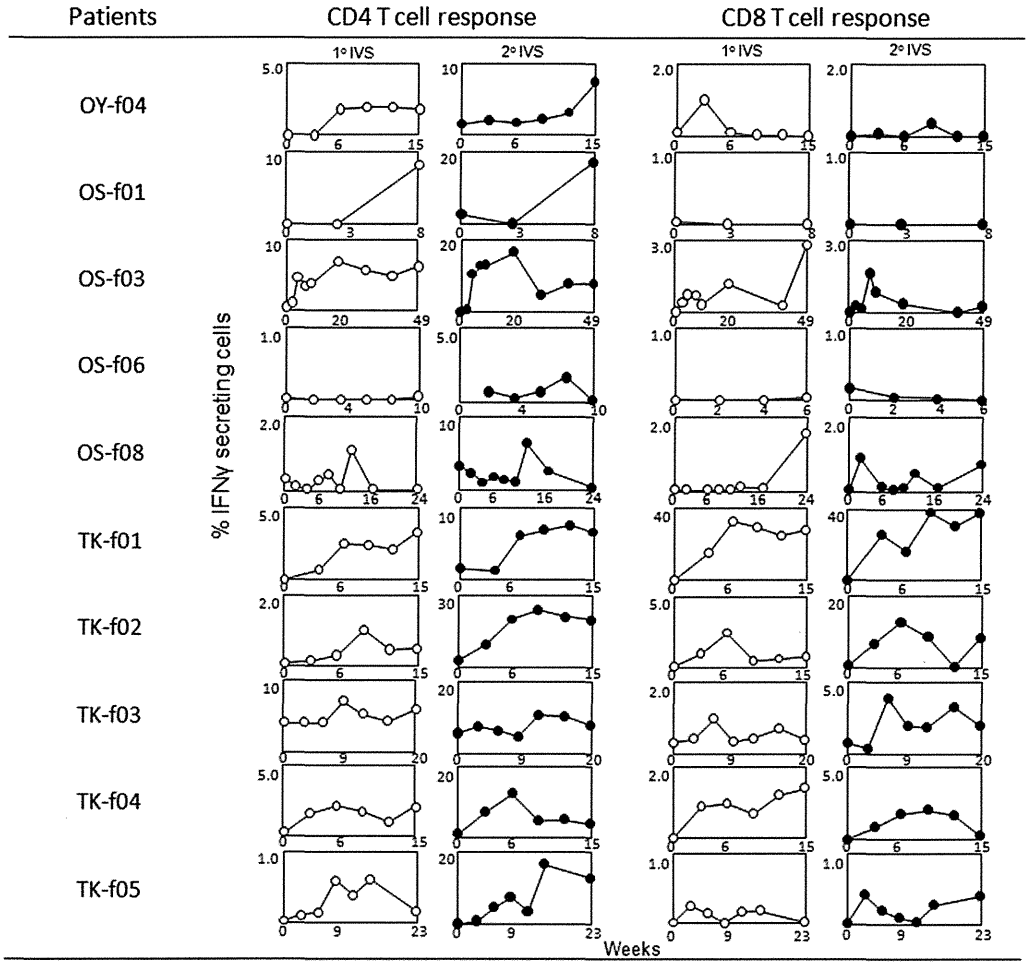

Figure 3. CD4 and CD8 T cell responses determined by the IFNγ capture assay. The net percentage of IFNγ-secreting cells of the total number of CD4 and CD8 T cells in cultures was plotted at the baseline and after each vaccination.

Clinical responses

Table 3 summarizes the immune and clinical responses in all patients. Stable disease (SD) was observed in three patients, including two patients with lung cancer and one patient with esophageal cancer. Lung cancer patient TK-f01 received a right middle lobectomy in October 2004, followed by postoperative adjuvant chemotherapy with Tegafur-Uracil (UFT) for 6 months. Since recurrence was detected in the left lung and a right hilar lymph node by CT scan in April 2007, he received three courses of combination chemotherapy with carboplatin and paclitaxel. As the tumor continued to grow despite the chemotherapy, he was enrolled in the study in June 2008. After initiating the vaccine, the tumor remained stable for 6 months and was classed as SD at the end of the sixth vaccination (Supporting Information Fig. 3*a*). The patient subsequently received another cycle of six vaccinations. However, the tumor started to grow after the eighth vaccination, consistent with an accelerated elevation in the serum CEA level. A greater than 20% increase in the sum of target lesion diameters was detected after the 11th vaccination, and this was evaluated as progressive disease (PD).

Lung cancer patient TK-f02 received a right upper lobectomy in January 2001, followed by postoperative adjuvant chemotherapy with UFT. Recurrence was noticed in March 2002. After that he received cryoablation surgery and chemotherapy including S-1 (oral fluoropyrimidine), combination chemotherapy with carboplatin and paclitaxel, Gefitinib and Erlotinib, one after the other. The nodule in the right middle lobe continued to grow, and the serum CEA level increased despite these therapies. Therefore, he was enrolled in the study in August 2008. After initiating the vaccine, growth of the tumor evaluated by CT scan, and the increase in the serum CEA level slowed down during the initial course of six vaccinations (Supporting Information Fig. 3*b*). Although a small nodule was detected as a new lesion in the left lower lobe after the fifth vaccination, another cycle of six vaccinations was given. During the second cycle of vaccinations, the sum of target diameters was almost unchanged from 24.8 to 27 mm (less than a 10% increase).

Esophageal cancer patient TK-f05 received surgery in January 2006, followed by two courses of postoperative adjuvant chemotherapy comprising CDDP and 5-fluorouracil. In July

Table 3. Study summary: Immune and tumor responses after vaccination with the NY-ESO-1f peptide

ID	IHC[1]		Antibody[2]		CD4[3]		CD8[3]		Clinical response[4]
	MHC class I	NY-ESO-1	Pre	Post	Pre	Post	Pre	Post	
OY-f04	+++	+++	−	+	−	++	−	++	PD
OS-f01	+++	+++	−	+	−	+++	−	−	PD
OS-f03	++	++	+++	+++	−	+++	−	++	PD
OS-f06	+++	+++	−	−	−	−	−	+	PD
OS-f08	++	+	−	++	+	++	−	++	PD
TK-f01	+++	++	−	++	−	++	−	+++	SD (→PD)
TK-f02	+++	++	−	++	−	++	−	++	PD (→SD)
TK-f03	+++	+	++	++	++	+++	+	++	PD
TK-f04	++	+++	−	++	−	++	−	++	PD
TK-f05	++	+	−	+++	−	+	−	+	SD (→PD)

[1]IHC was performed using EMR8–5 mAb for MHC class I and E957 mAb for NY-ESO-1. IHC-positive cells: +++ > 50%; 50% ≥ ++ > 25%; 25% ≥ + > 5%; 5% ≥ −. [2]Antibody response was determined by ELISA (see Material and Methods) using O.D. values for NY-ESO-1f peptide at a serum dilution of 1:25 and for NY-ESO-1 protein at a serum dilution of 1:100 for seronegative patients or 1:1,600 for seropositive patients. Antibody response against protein is shown. Antibody: +++ > 2; 2 ≥ ++ > 0.5; 0.5 ≥ + > 0.1; 0.1 ≥ −. [3]CD4 and CD8 T cell responses were determined by IFNγ capture assay in once *in vitro* stimulation (1°IVS). The response was confirmed by that of two times *in vitro* stimulation (2°IVS). IFNγ-positive cells: +++ > 5%; 5% ≥ ++ > 1%; 1% ≥ + > 0.1%; 0.1 ≥ −. [4]Clinical response was assessed according to RECIST criteria at Weeks 20–22 and after additional injections (described in parentheses) in patients with a prolonged disease course.

2008, enlarged para-aortic lymph nodes were observed in the upper abdomen. The patient received combination chemotherapy with docetaxel, cisplatin and 5-fluorouracil (DCF); however, the lymph node metastases were exacerbated. Therefore, he was enrolled in the study in March 2009. Uptake of fluorine-18-labeled FDG (fluorodeoxyglucose) in para-aortic lymph node measured as the maximum standardized uptake value by PET (positron emission tomography) was initially increased from 12.7 in February 2009 to 14.7 in May 2009, but gradually deceased to 10.3 in September 2009 (Supporting Information Fig. 3*c*). The CT scan showed that some low-density areas corresponding to necrotic change appeared in the lymph nodes (Supporting Information Fig. 3*c*). The clinical response was evaluated as SD after the sixth vaccination, and he received another cycle of vaccinations. However, bone metastasis in the sternum was suspected by PET-CT (Supporting Information Fig. 3*c*, red arrow). Finally, new lesions appeared in the lung in November and he was withdrawn from the study.

Discussion

In our study, we immunized patients with NY-ESO-1-expressing tumors by injecting the NY-ESO-1f peptide (600 μg) mixed with Picibanil OK-432 (0.2 KE) and Montanide ISA-51 (1.25 ml) subcutaneously once every 3 weeks for six doses and evaluated the safety and immunological responses. The study population consisted of ten patients, including six patients with esophageal cancer, three patients with non-small-cell lung cancer and one patient with gastric cancer. As vaccine-related adverse events Grade 1 fever, Grade 1 and 2 injection-site reactions and Grade 2 induration were observed. The treatment was considered to be well tolerated. Vaccination with the NY-ESO-1f peptide with Picibanil and Montanide resulted in an increase or induction of an NY-ESO-1 antibody response in nine of ten patients immunized. An increase or induction of CD4 and CD8 T cell responses was also observed in nine of ten patients. These findings confirmed the immunogenicity of the NY-ESO-1f peptide. Furthermore, three patients, including two patients with lung cancer and a patient with esophageal cancer, showed SD.

Recently, the advantage of synthetic long peptides over short peptides for use as vaccines has been acknowledged.[19] Long peptides do not bind to MHC class I molecules directly, and the antigen is presented after processing by dendritic cells. Therefore, use of long peptides prevents the antigen peptides from direct binding to MHC class I molecules on nonprofessional antigen-presenting cells such as B cells and T cells, which may cause transient activation of CTLs followed by their subsequent anergy in the absence of appropriate costimulatory signals.[26]

Furthermore, because Th cells licensed DCs for their efficient antigen presentation and stimulation capacity, introduction of a Th epitope into the vaccine or physical linking of Th and CTL epitope peptides facilitated increased immunogenicity of CTL vaccines.[27,28] Interestingly, Th and CTL epitopes are sometimes located in close proximity or are even overlapped, in the molecules, for example, in the case of the human papillomavirus[29] and Her-2/neu.[30] Zeng *et al.*[31] reported that NY-ESO-1 157–170 (SLLMWITQCFLPVF) was recognized by both NY-ESO-1-reactive CD4 and CD8 T cells. The synthetic long peptide containing overlapping CD4 and CD8 T cell epitope sequences in the antigens is expected to generate both CD4 and CD8 T cell responses as a vaccine.

Recently, we identified regions II (73–114) and III (121–144) in the NY-ESO-1 molecule that were frequently recognized by either CD4 or CD8 T cells irrespective of the patients'

HLA type.[16] Moreover, the most dominant peptide region (91–108) eliciting an antibody response was also included in region II.[15] Our study showed that a long peptide, NY-ESO-1f, spanning a peptide region 91–110 was immunogenic and induced antibody, CD4 and CD8 T cell responses in patients.

In our study, Picibanil® OK-432 was chosen as an adjuvant. Picibanil is dried penicillin-treated *Streptococcus pyogenes*, which has been shown to activate the immune cells of both the innate and adaptive immune system.[32,33] Montanide® ISA-51[34], which causes inflammation at the injection site and is believed to be helpful in attracting immune cells, was used as the vehicle to deliver the vaccine containing NY-ESO-1f peptide and Picibanil® OK-432. Montanide® ISA-51 also forms a local depot that allows persistence of antigens resulting in prolonged immune activation. This formula induced Grade 1 fever (38–39°C) in six of ten patients that subsided within several days without any medication. In addition, the vaccine induced a robust skin reaction when it was injected close to the dermis of the skin. The reaction caused erythema and induration at the site of the vaccine injection within 48–72 hr. The intensity of the skin reaction was augmented by repeated vaccinations as shown in TK-f02 (Supporting Information Fig. 1), suggesting the reaction was a delayed-type hypersensitivity reaction against Picibanil or the NY-ESO-1f peptide in this patient. The induration was sustained during the course of the treatment, but it subsided gradually. Surgical specimens for histological examination were not available in our study.

The NY-ESO-1f peptide vaccine elicited humoral, CD4 and CD8 T cell responses in the immunized patients (Table 3). The increase or induction of an NY-ESO-1 antibody response was observed in nine of ten immunized patients. The sera from NY-ESO-1f peptide-immunized patients reacted with NY-ESO-1 protein as well as the NY-ESO-1f peptide, suggesting elicitation of an antibody response by a long peptide vaccine including a dominant B cell epitope. The increase and induction of CD4 and CD8 T cell responses were also detected after NY-ESO-1f peptide vaccination in nine of ten patients. Although the number of patients was small, the responses were comparable or even stronger in terms of the frequency and characteristics of the immune response, when compared with various preparations of NY-ESO-1 protein vaccine such as NY-ESO-1/ISCOMATRIX,[35] NY-ESO-1 vaccinia/fowlpox,[36] NY-ESO-1/CpG/Montanide[11,37] and CHP-NY-ESO-1 vaccines.[16]

It has been reported that vaccination with the NY-ESO-1 protein with CpG and Montanide elicited detectable CD8 T responses in half of the immunized patients (9/18), and vaccine-induced CD8 T cells mostly recognized NY-ESO-1 81–110 restricted by either HLA-B35 or HLA-Cw3.[11,37] Consistently, we also observed that vaccination with the NY-ESO-1f peptide elicited CD8 T responses in patients OS-f08, TK-f01, TK-f03, TK-f04 and TK-f05, who were shown to be positive for HLA-B35 and/or HLA-Cw3 (Table 2). In addition, NY-ESO-1f peptide vaccination induced CD8 T responses in patients OY-f04, OS-f03, OS-f06 and TK-f02, who were shown to be negative for HLA-B35 and HLA-Cw3. Thus, it is not necessary for the NY-ESO-1f peptide vaccine to exclude patients who are negative for HLA-B35 and Cw3.

B35-binding peptide epitopes 94–102 and 94–104 and Cw3-binding peptide epitopes 92–100 and 96–104 have been described.[38,39] Analysis of the CD8 T cell response using OLPs revealed that the NY-ESO-1f peptide (NY-ESO-1 91–110) vaccine elicited a response to peptide 16 (NY-ESO-1 91–108) in all six patients analyzed with or without B35 and/or Cw3. The vaccine elicited a CD8 T cell response to peptide 15 (NY-ESO-1 85–102) in two of six patients to a lesser extent. Although a full-length protein vaccine can potentially induce multiple immune responses restricted to different HLA molecules in a patient, the presence of an immunodominant epitope may shift the response to a dominant one. If the NY-ESO-1f peptide is a subdominant epitope in a given patient, the peptide can be efficiently recognized by T cells in the absence of a dominant epitope. The fact that NY-ESO-1f peptide vaccine elicited CD8 T cell responses in patients with various HLA types suggests the advantage of a long peptide over the whole protein for vaccination. Extending our study using a single NY-ESO-1f peptide as a vaccine, we are now conducting a clinical trial of a cancer vaccine using multiple overlapping long peptides spanning NY-ESO-1 79–173 that includes highly immunogenic regions II (73–114) and III (121–144).

In our study, we observed SD in three of ten patients enrolled, including two patients with lung cancer and a patient with esophageal cancer (Supporting Information Fig. 3). Integrated antibody, CD4 and CD8 T cell responses were detected in all of these patients. Although patient TK-f01 expressed both HLA-B35 and Cw3, patients TK-f02 and TK-f05 expressed none of these antigens.

It is now accepted that an immune-related tumor response should be evaluated by different criteria from that for a tumor response induced by cytotoxic agents.[40] Clinical response resulting from immunotherapy can be appreciated generally after an initial increase in tumor volume sometimes associated with the appearance of new lesions evaluated as PD by the Response Evaluation Criteria in Solid Tumors (RECIST) or WHO criteria. Thus, immune-related response criteria (irRC) were proposed recently.[41] For TK-f02 in our study, the increase in tumor diameter measured by CT images was less than 20% of the initial tumor burden, and a reduced increase in serum CEA level was observed during the initial course of six vaccinations. However, a small new lesion was noticed in the left lower lobe after the fifth vaccination (Supporting Information Fig. 3*b*). Patient TK-f02 was PD according to the RECIST criteria, but irSD according to irRC. As both CD4 and CD8 T cell responses were detected even after the first NY-ESO-1f peptide vaccination, we decided to give another cycle of six vaccinations to this patient, resulting in sustained SD with good quality of life.

In summary, the NY-ESO-1f peptide is a dominant region in the NY-ESO-1 molecule that includes multiple epitopes frequently recognized by antibody, CD4 and CD8 T cells.

Therefore, the use of the NY-ESO-1f peptide as a cancer vaccine will practically allow inclusion of most, if not all, patients into a study irrespective of their HLA type. The finding that the NY-ESO-1f peptide vaccine caused little toxicity and strong humoral and cellular immune responses suggests the usefulness of long peptide vaccines in the clinical management of cancer patients.

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