cancer (12-17). Research has found that IL-17 promotes tumor growth through angiogenesis and inflammation (18), and several other studies have demonstrated that IL-17 contributes to reduced tumor growth by promoting dendritic cell, cytotoxic T cell (CTL), and NK cell trafficking to, and retention within, the tumor microenvironment (19,20). Therefore, whether these cells promote tumor growth or regulate antitumor responses remains controversial. In the case of human gastric cancer, there are several reports concerning the prevalence of Th17 cells in the tumor microenvironment, tumor-draining lymph nodes and peripheral blood (12,13). In our previous study, the expression level of IL-17 mRNA in gastric tumors was associated with the depth of tumor invasion, lymphovascular invasion and lymph node involvement suggesting that IL-17 is clearly associated with tumor progression (21). However, since no previous studies have been carried out concerning the proportion of IL-17 in the peritoneal cavity in human gastric cancer patients, we hypothesized that the expression level of IL-17 mRNA in peritoneal lavage may be involved in the development of peritoneal carcinomatosis in gastric cancer.

In the present study, we quantitatively investigated expression of IL-17 messenger RNA (mRNA) in the peritoneal lavage of gastric cancer patients who underwent curative resection. The association of IL-17 expression levels with clinicopathological factors and prognosis was also assessed. Since IL-2 and IL-12 have been used for intraperitoneal immunotherapy in patients with various types of cancers, including gastric cancer (22-25), the possibility of IL-17 as a therapeutic target for patients with gastric cancer was also investigated.

Materials and methods

Patients. Included in the present study was a series of 114 patients (80 men and 34 women) with gastric cancer who underwent surgery at Wakayama Medical University Hospital (WMUH) from 2003 to 2006. At the beginning of the operation, we examined tumor metastases in the abdominal cavity. When it was thought that curative resection was possible, we performed gastrectomy with lymphadenectomy. We performed gastrectomy in 114 patients with gastric cancer. Seventy-nine patients underwent surgically curative resection and 35 underwent non-curative resection. None of the patients received anticancer therapy prior to surgery. Individuals with autoimmune disease, inflammatory bowel disease or viral infections were excluded. The clinicopathological characteristics of the 114 patients are summarized in Table IA. Clinical stages of the tumors were determined according to the International Union Against Cancer's TNM classification for gastric cancer. After surgery, all patients underwent a follow-up, with the median follow-up at analysis being 61 months (range, 1.3-98.5) for all patients. Every 3 to 6 months, physical examination, blood chemistry, including carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9, and computed tomography were performed for each patients. Written informed consent was obtained from all patients before participation in the present study. In addition, the local ethics committee of WMUH approved the study.

Preoperative peritoneal wash examination. At the beginning of each operation, 100 ml saline was introduced into the Douglas cavity and aspirated by gentle stirring. These washes were centrifuged at 1,800 rpm for 5 min to collect intact cells. A part of each peritoneal wash was examined cytopathologically after conventional Papanicolaou staining.

RNA extraction and DNA synthesis. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) followed by RNase-Free DNase Set treatment (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using a reverse transcription system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Samples were stored at -80°C until use.

Quantitative real-time RT-PCR. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed with isolated total RNA (1 μg) on the LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany). The following oligonucleotide primers and hybridization probes were used: human IL-17 (GenBank accession no. NM 002190; 53-231 bp): sense, 5'-CTGGGAAGACCTCA TTGG-3'; antisense, 5'-CCTTTTGGGATTGGTATTGG-3'; fluorescein-labeled probe, 5'-TCCTCAGAATTTGGGC ATCCTGGATTTC-3'; and LC Red 640-labeled probe, 5'-TGGGATTGTGATTCCTGCCTTCACTATGG-3'; human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GenBank accession no. NM 002046; 746-1052 bp): sense, 5'-TGAACGGGAAGCTCACTGG-3'; antisense, 5'-TCC ACCACCCTGTTGCTGTA-3'; fluorescein-labeled probe, 5'-TCAACAGCGACACCCACTCCT-3'; and LC Red 640-labeled probe, 5'-CACCTTTGACGCTGGGGCT-3'. Primers and probes were designed by Nihon Gene Research Laboratories, Inc. (Miyagi, Japan). After 10 min of initial denaturation at 95°C, the cycling protocol entailed 40 cycles of denaturation at 95°C (10 sec), annealing at 62°C (15 sec) and elongation at 72°C (8 sec). For GAPDH, the thermocycling protocol was the same, except that annealing was performed at 55°C (15 sec) and 50 cycles were run. On each run, all samples were quantified according to the LightCycler software program, version 3.8 (Roche Molecular Biochemicals). The levels of mRNA for IL-17 were corrected with GAPDH housekeeping control amplifications. We used the following for quantitative RT-PCR analysis: IL-17 ratio = IL-17 value/ GAPDH value x 10^4.

Determination of the cut-off value. The cut-off value of the IL-17 mRNA ratio was determined as the median value based on the quantified values of 114 samples in the present study. The cut-off value was 1.22.

Immunohistochemistry and quantitative microscopy. Sections (4 μm) were prepared from paraffin-embedded blocks derived from gastric tumors. Sections were deparaffinized in xylene and graded alcohols, and rinsed in phosphate-buffered saline. Antigen retrieval from the tissues was carried out by autoclaving the tissues in 0.01 M citrate buffer (pH 6.0) at 100°C for 10 min. The antibody used was goat anti-IL-17 (dilution at 10 μg/ml; R&D Systems, Minneapolis, MN, USA). The antibodies were incubated overnight at 4°C. The immunocomplex was visualized by a polymer envision method, EnVision™+ Kit (DakoCytomation, Glostrup, Denmark). For quantification

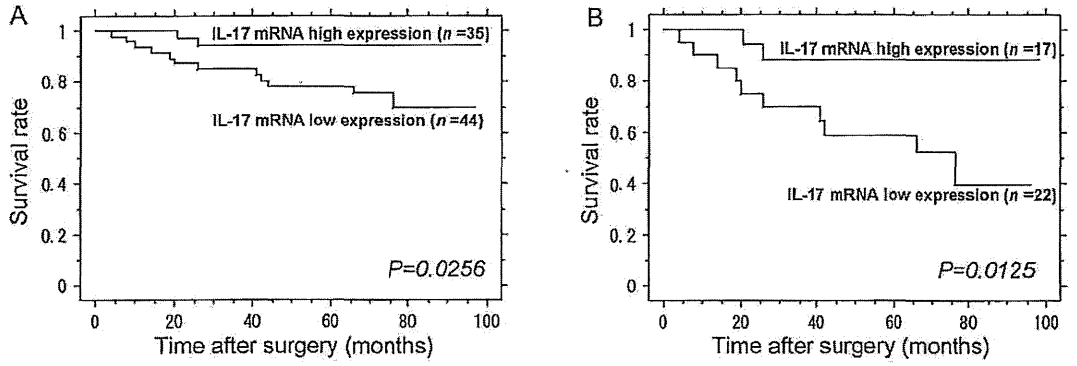

Figure 1. (A) Overall survival curves of the 79 gastric cancer patients who underwent curative R0 resection stratified according to IL-17 mRNA high or low expression in peritoneal lavage as determined by real-time RT-PCR. A significant difference in survival was noted between the IL-17 mRNA high expression and IL-17 mRNA low expression groups (P=0.0256; log-rank test). (B) The overall survival of the 79 patients who underwent curative resection with clinical stage II/III tumors according to IL-17 mRNA high or low expression in peritoneal lavage as determined by real-time RT-PCR. The IL-17 mRNA low expression group had a significantly poorer prognosis than the high expression group among the clinical stage II/III patients (P=0.0125, log-rank test).

of IL-17-positive cells, highly positive areas were initially identified by scanning tumor sections using light microscopy. Data were obtained by manually counting positively stained cells in five separate areas of intratumoral regions under x400 high power magnification. The regions were counted by a pathologist who had no knowledge of the other clinicopathological features and survival outcomes.

Flow cytometry. For intracellular molecule measurements, cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 5 h in the presence of GolgiPlug (BD Biosciences, San Diego, CA, USA). Then, the cells were harvested and stained with PerCP-Cy5.5-conjugated anti-CD3 mAb, FITC-conjugated anti-CD4 mAb, FITC-conjugated anti-CD8 mAb and FITC-conjugated anti-γδ TCR mAb (BD Biosciences) for 30 min on ice. For intracellular staining, after fixation and permeabilization using BD Cytofix/Cytoperm (BD Biosciences), the cells were stained with PE-conjugated anti-IL-17 mAb (BD Biosciences) for 30 min at 4˚C. After washing, the cells were analyzed by FACSCalibur, using CellQuest (BD Biosciences).

Statistical analysis. The Mann-Whitney test and the Kruskal-Wallis test were used to determine statistical significance between the covariates. The Cox proportional hazards model was used to compute the univariate and multivariate hazards ratios for the study parameters. Survival curves were computed using the Kaplan-Meier method and compared by means of the log-rank test. The survival curve was calculated from the date of surgery. In Fig. 3, we used the Spearman rank correlation coefficient. All statistical analyses were performed with StatView 6.0 (Abacus Concepts, Inc., Berkeley, CA, USA) statistical software program. A value of $P<0.05$ was considered to indicate a statistically significant result.

Results

Relationship between IL-17 mRNA and clinicopathological factors. To evaluate the biological significance of IL-17 expression in peritoneal lavage from patients with gastric cancer, the association between mRNA expression levels of IL-17 and clinicopathological factors was investigated. In all patients, the IL-17 mRNA expression level in the peritoneal lavage increased according to the depth of tumor invasion and peritoneal metastases, while the expression level was not associated with cytologic examination (Table IA). In the patients who underwent curative resection, IL-17 mRNA expression levels in peritoneal lavage increased according to the depth of tumor invasion (Table IB). On the other hand, no significant association was recognized between the expression level of IL-17 mRNA and histological type, lymph node metastases, lymphatic invasion, vessel invasion, clinical stage or tumor size.

Correlation between patient survival and IL-17 mRNA expression in peritoneal lavage. Kaplan-Meier survival curves indicated the overall survival of gastric carcinoma patients stratified according to the results of the IL-17 mRNA expression status in peritoneal lavage. The survival curves of all 114 patients displayed no significant difference between the IL-17 mRNA low expression group and the IL-17 mRNA high expression group (data were not shown). Importantly, however, based on the survival curves, among the 79 patients who underwent curative R0 resection, the patients in the IL-17 mRNA low expression group (n=44) had a significantly poorer prognosis when compared with the patients in the IL-17 mRNA high expression group (n=35) (Fig. 1A; $P<0.05$). During the median 61 months of postoperative surveillance, 14 (17.8%) of the 79 patients who underwent curative resection died, and 12 (85.7%) of these 14 patients developed peritoneal metastasis. Regarding peritoneal recurrence, 10 (22.7%) of the 44 cases in the IL-17 mRNA low expression group developed peritoneal metastases, while 2 (5.7%) of the 35 cases in the IL-17 mRNA high expression group developed peritoneal metastases.

Correlation between survival and IL-17 mRNA in peritoneal lavage in advanced gastric cancer. Among the patients who underwent curative resection with clinical stage II/III tumors, the prognosis of the IL-17 mRNA low group was significantly

Table I. Clinicopathological data and IL-17 mRNA expression of the 114 patients and the 79 patients who underwent curative resection.

A, Data of the 114 patients

Factor	No. of patients	Expression of IL-17 mRNA[a]	P-value
Age (years)			
≤65	50	2.11±0.648	0.448
>65	64	1.57±0.399	
Gender			
Male	80	1.75±0.415	0.718
Female	34	1.93±0.724	
Depth of tumor invasion			
T1	30	1.00±0.523	0.023[c]
T2	13	1.49±0.130	
T3	31	2.44±0.809	
T4	40	2.51±0.582	
Lymph node metastasis			
N0	43	2.25±0.681	0.477
N1	28	1.26±0.581	
N2	21	1.61±0.708	
N3	22	1.84±0.894	
Histological type			
Differentiated	54	2.08±0.601	0.387
Undifferentiated	60	1.55±0.423	
Lymphatic invasion			
Negative	37	1.90±0.659	0.338
Positive	77	1.76±0.432	
Vessel invasion			
Negative	57	1.83±0.544	0.292
Positive	57	1.78±0.479	
Peritoneal metastasis			
Negative	100	2.07±0.439	0.031[d]
Positive	14	7.21±0.290	
Cytologic examination			
Negative	91	1.22±0.591	0.801
Positive	23	1.89±0.404	
Stage[b]			
I	40	1.95±0.660	0.412
II	18	2.29±0.109	
III	21	1.38±0.784	
IV	33	1.64±0.531	
Tumor size (cm)			
≤5	60	1.75±0.516	0.059
>5	54	1.87±0.506	

Table I. Continued.

B, Data of the 79 patients who underwent curative resection

Factor	No. of patients	Expression of IL-17 mRNA[a]	P-value
Age (years)			
≤65	35	2.01±0.781	0.329
>65	44	1.72±0.527	
Gender			
Male	58	1.80±0.517	0.975
Female	21	1.93±0.724	
Depth of tumor invasion			
T1	30	1.00±0.523	0.020[c]
T2	13	1.49±0.130	
T3	23	2.39±0.927	
T4	13	2.90±0.124	
Lymph node metastasis			
N0	43	2.25±0.681	0.307
N1	20	1.48±0.700	
N2	10	1.90±0.106	
N3	6	3.26±0.284	
Histological type			
Differentiated	41	2.31±0.719	0.613
Undifferentiated	38	1.33±0.524	
Lymphatic invasion			
Negative	34	1.72±0.690	0.118
Positive	45	1.95±0.610	
Vessel invasion			
Negative	49	1.72±0.597	0.087
Positive	30	2.05±0.708	
Stage[b]			
I	40	1.95±0.660	0.497
II	18	2.29±0.109	
III	21	1.44±0.819	
Tumor size (cm)			
≤5	54	1.62±0.509	0.097
>5	25	2.36±0.943	

[a]Expression of mRNA for IL-17 was corrected with GAPDH housekeeping control amplifications. Values represent mean ± SEM. [b]Stage according to the TNM classification for gastric cancer (UICC). [c]P-value of Kruskal-Wallis test as appropriate. [d]P-value of Mann-Whitney test as appropriate.

poorer than that of the patients in the IL-17 mRNA high group (Fig. 1B; P<0.05). From the point of view of the depth of the invasion, patients in the IL-17 mRNA low group had significantly poorer outcome than those in the IL-17 mRNA high group for patients in the T2/3/4 subgroups (Fig. 2A; P<0.05). In the T4 subgroup, patients with IL-17 mRNA low expression in peritoneal lavage had a significantly poorer survival than those with IL-17 mRNA high expression (Fig. 2B; P<0.05).

Preoperative peritoneal wash assay as an independent prognostic factor. We evaluated prognostic factors in the 79 patients who underwent curative R0 resection. With the overall

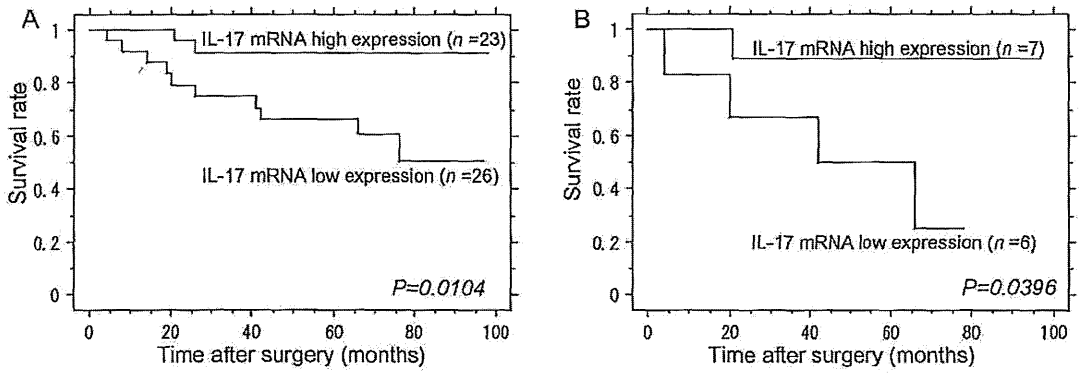

Figure 2. Overall survival curves of the 79 gastric cancer patients who underwent curative resection with pT2/3/4 stage tumors stratified according to IL-17 mRNA high or low expression in peritoneal lavage. (A) Overall survival of the 79 patients subdivided according to IL-17 mRNA high or low expression in peritoneal lavage with pT2/3/4 stage tumors. The IL-17 mRNA low expression group had a significantly poorer prognosis than the high expression group in the combined pT2/3/4 subgroup (P=0.0104; log-rank test). (B) Overall survival of the 79 patients with pT4 stage tumors subdivided according to IL-17 mRNA high or low expression in peritoneal lavage. The IL-17 mRNA low expression group had a significantly poorer prognosis than the high expression group among the pT4 patients (P=0.0396; log-rank test).

Table II. Univariate and multivariate analysis of the overall survival for the 79 patients who underwent R0 curative resection.

	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (years)						
≤65 vs. >65	1.62	0.542-4.840	0.388	-	-	-
Gender						
Male vs. female	1.07	0.335-3.438	0.906	-	-	-
Lymph node metastasis						
Negative vs. positive	9.98	1.296-76.95	0.027	2.94	0.175-49.39	0.454
Serosal invasion						
Negative vs. positive	9.04	2.014-40.53	0.004	1.56	0.215-11.35	0.659
Lymphatic invasion						
Negative vs. positive	9.98	1.296-76.95	0.027	1.05	0.046-23.79	0.976
Vessel invasion						
Negative vs. positive	10.38	2.320-46.42	0.0022	4.16	0.459-37.68	0.205
Histological type						
Differentiated vs. undifferentiated	2.16	0.720-6.497	0.169	-	-	-
Tumor size (cm)						
≤5 vs. >5	7.32	2.289-23.40	0.0008	4.61	1.19-17.78	0.027
IL-17 mRNA expression						
Low expression vs. high expression	4.69	1.049-20.99	0.043	7.91	1.65-38.03	0.0098

CI, confidence interval.

survival as an endpoint, lymph node metastasis, serosal invasion, lymphatic invasion, vessel invasion, tumor size and IL-17 mRNA expression were found to be significant as prognostic factors by univariate analysis. Moreover, when multivariate analysis was performed with these six covariates and the same endpoint, IL-17 mRNA low expression in peritoneal lavage and tumor size were found to be independent significant predictive factors for prognosis (Table II; HR, 7.91; 95% CI, 1.65-38.03; P=0.0098).

Correlation between IL-17-positive cells in primary tumor tissues and IL-17 mRNA expression in peritoneal lavage. To examine the correlation in IL-17 production between the level in peritoneal lavage and in the primary tumor tissues,

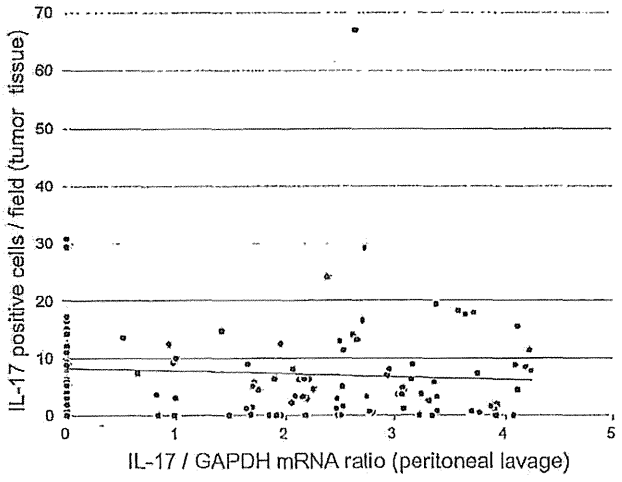

Figure 3. Correlation between IL-17-positive cells in tumor tissues and IL-17 mRNA expression in peritoneal wash. Immunohistochemical staining for IL-17 in primary tumor tissues was performed in the same patient samples whose peritoneal lavage was examined for IL-17 mRNA. The longitudinal axis shows IL-17-positive cells per field in the tumor tissues. The horizontal axis shows the IL-17/GAPDH mRNA ratio in peritoneal lavage.

we performed immunohistochemical staining for IL-17 in the primary tumors in the same patient samples. In the primary tumor tissues, IL-17 immunoreactive cells were detected in the cytoplasm of mononuclear cells; however, none of the tumor cells were stained for IL-17. IL-17-producing cells in the tumor tissues were 7.30±0.82 (mean ± SE) per field. There was no correlation between the number of IL-17-positive cells in the tumor tissues and IL-17 mRNA expression in the peritoneal wash (r=0.092; P=0.329) (Fig. 3).

Analysis of IL-17-producing cells in peritoneal lavage. Immunohistochemical staining of peritoneal wash revealed that mononuclear cells were stained for IL-17. However, neither tumor cells nor mesothelial cells were stained for IL-17. To identify which mononuclear cells produced IL-17 in the peritoneal lavage, we performed flow cytometric analysis using anti-IL-17, -CD3, -CD4, -CD8 and -γδ TCR antibodies. $CD3^+$ T cells produced IL-17, while γδ T cells did not produce IL-17 (Fig. 4A). $CD4^+$ T cells mainly produced IL-17, and a small population of $CD8^+$ T cells also produced IL-17. The mean percentage of IL-17-positive $CD8^+$ T cells among the total IL-17-positive cell population was only 27.6±4.85% (n=5), and in contrast, IL-17-positive $CD4^+$ T cell population was 72.2±4.86% (n=5). Representative flow cytometry analysis is shown in Fig. 4B.

Discussion

In the present study, we demonstrated that in patients who underwent R0 resection, the prognosis of patients in the IL-17 mRNA low expression group was significantly poorer than those in the high expression group. This is the first study evaluating the prognostic value of IL-17 detection by real-time RT-PCR in peritoneal lavage as a valuable prognostic factor in gastric cancer.

IL-17 was originally identified as a proinflammatory cytokine that induces neutrophils, and previous studies also have shown that inflammation is linked to cancer development and progression. It has recently been reported that the levels of IL-17-producing cells are significantly increased in tumor tissues, peripheral blood, malignant ascites fluid, and malignant pleural effusion from a variety of cancer patients (13,14,26-28). Despite recent advances in our understanding of the function of Th17 cells in humans, very little is known about their prevalence and tumor immunosurveillance.

In mice, overexpression of IL-17 by gene transduction into tumor cells promoted tumor growth through angiogenesis (18), but seemingly in contrast, IL-17 also suppressed tumor growth

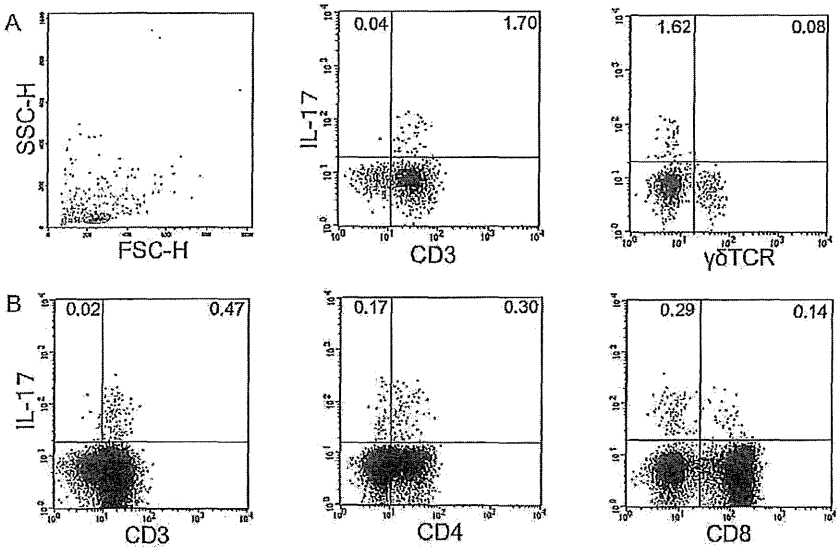

Figure 4. Flow cytometric analysis using anti-IL-17, -CD3, -CD4, -CD8 and -γδ TCR antibodies to identify IL-17-producing cells in peritoneal washes. (A) Cells were stained with PE-anti-IL-17 mAb, PerCP-anti-CD3 mAb and FITC-anti-γδ TCR mAb after stimulation with PMA and ionomycin for 5 h. (B) Cells were stained with PE-anti-IL-17 mAb, PerCP-anti-CD3 mAb, FITC-anti-CD4 mAb and FITC-anti-CD8 mAb after stimulation with PMA and ionomycin for 5 h. Representative flow cytometry plots using peritoneal washes from subjects with cytology class II.

via a T-cell-dependent mechanism (19). Whether IL-17 promotes tumor growth or regulates antitumor responses remains controversial.

In humans, there are several reports concerning intratumoral expression of IL-17 and its prognostic role in several cancer types such as hepatocellular carcinoma (HCC), colon, esophageal and gastric cancer. In HCC, colon, and prostate cancer patients, intratumoral IL-17-positive cells were found to be correlated with poor survival (17,29,30). Our previous study showed that Th17 cells infiltrated the tumor and secreted IL-17 in the tumor microenvironment, leading to tumor progression through angiogenesis and neutrophil infiltration in patients with gastric cancer. In the present study, we hypothesized that IL-17 promotes tumor progression in the peritoneal cavity, based on our previous study suggesting that IL-17 is related to tumor progression in the tumor microenvironment. We quantitatively analyzed the expression levels of IL-17 mRNA in peritoneal lavage from gastric cancer patients. Based on the survival curves, among the 79 patients who underwent R0 resection, the patients in the IL-17 mRNA low expression group had a significantly poorer prognosis than the patients in the IL-17 mRNA high expression group. This result was contradictory to our hypothesis. This discrepancy may be explained by the difference in the impact of IL-17 on tumor progression in the thoracoabdominal cavity and in tumor tissue. In fact, in the present study, there were no correlations noted between primary tumor tissues and peritoneal wash in terms of IL-17 expression. In lung cancer, Ye *et al* (26) reported that patients with a higher proportion of Th17 cells in malignant pleural effusion exhibited significantly longer overall survival than patients with a lower proportion of Th17 cells. Similarly, in ovarian cancer, the expression of IL-17 in ascites was analyzed, and patients with a higher IL-17 expression in ascites had a significantly lower death hazard than those with a lower IL-17 expression (28).

Most recently, it has been reported that $CD8^+$ T cells that produce IL-17 (Tc17 cells) are abundant in gastric cancer tissue, and the percentage of Th17 cells is relatively lower than that of Tc17 cells in tumors. The intratumoral Tc17 cell percentage was significantly associated with tumor progression and poor prognosis (31). In the present study, flow cytometric analysis showed that $CD4^+$ Th17 cells predominantly produced IL-17 in the peritoneal lavage; however, the percentage of Tc17 cells was lower than that of Th17. This suggests that IL-17-producing T cells are different between tumor tissue and the abdominal cavity, and the potential role of IL-17 could also be different in the tumor microenvironment between tumor tissue and the abdominal cavity.

There is another reason why the results of the present study were in conflict with our expectations. This may be because the role of IL-17 is different before and after the tumor is established. In the present study, the expression levels of IL-17 were significantly higher in peritoneal carcinomatosis-positive cases than those of negative cases, while they were not associated with cytologic examination (Table IA). Once tumor cells attach to the peritoneum, IL-17 may play a role as a tumor growth cytokine through angiogenesis to a greater exent than its role in regulating antitumor responses. Our results suggest that endogenous IL-17 plays different roles before tumor attachment versus in established tumor growth. Furthermore, in the abdominal cavity, previous studies indicate that peritoneal mesothelial cells secrete various cytokines and growth factors, such as IL-6, IL-8, IL-1α and β, granulocyte colony stimulating factor (G-CSF), as well as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2. These results indicate that peritoneal mesothelial cells are one of the central elements of the cytokine network controlling disease processes in the abdominal cavity. Kryczek *et al* suggested that, in the peritoneal cavity, IL-17 is positively associated with INF-γ effector T cells and Th1-type chemokines, CXCL9 and CXCL10, but not with Th2-type chemokines, CXCL12 and CCL22, in ovarian cancer ascites. Mechanistically, Th17 cell-derived IL-17 and INF-γ were found to synergistically induce the production of CXCL9 and CXCL10 and in turn promote effector T-cell migration (28). Thus, IL-17 may function as a polyfunctional cytokine profile in human tumors. There is no doubt that the role of IL-17 is highly complicated, and it remains controversial whether IL-17 promotes tumor growth or regulates the antitumor response.

In conclusion, IL-17 mRNA expression in peritoneal lavage detected by real-time RT-PCR is a reliable prognostic factor for patients with curative resection in gastric cancer. Low IL-17 gene expression in the peritoneal cavity may correlate with cancer development in the peritoneal cavity and poor prognosis in patients with gastric cancer.

References

1. Takahashi I, Matsusaka T, Onohara T, *et al*: Clinicopathological features of long-term survivors of scirrhous gastric cancer. Hepatogastroenterology 47: 1485-1488, 2000.
2. Ito S, Nakanishi H, Kodera Y, Mochizuki Y, Tatemastu M and Yamamura Y: Prospective validation of quantitative CEA mRNA detection in peritoneal washes in gastric carcinoma patients. Br J Cancer 93: 986-992, 2005.
3. Bonenkamp JJ, Songun I, Hermans J and van de Velde CJ: Prognostic value of positive cytology findings from abdominal washings in patients with gastric cancer. Br J Surg 83: 672-674, 1996.
4. Kodera Y, Nakanishi H, Yamamura Y, *et al*: Prognostic value and clinical implications of disseminated cancer cells in the peritoneal cavity detected by reverse transcriptase-polymerase chain reaction and cytology. Int J Cancer 79: 429-433, 1998.
5. Fujii S, Kitayama J, Kaisaki S, *et al*: Carcinoembryonic antigen mRNA in abdominal cavity as a useful predictor of peritoneal recurrence of gastric cancer with serosal exposure. J Exp Clin Cancer Res 21: 547-553, 2002.
6. Tokuda K, Natsugoe S, Nakajo A, *et al*: Clinical significance of CEA-mRNA expression in peritoneal lavage fluid from patients with gastric cancer. Int J Mol Med 11: 79-84, 2003.
7. Boku T, Nakane Y, Minoura T, *et al*: Prognostic significance of serosal invasion and free intraperitoneal cancer cells in gastric cancer. Br J Surg 77: 436-439, 1990.
8. Kodera Y, Nakanishi H, Ito S, *et al*: Quantitative detection of disseminated free cancer cells in peritoneal washes with real-time reverse transcriptase-polymerase chain reaction: a sensitive predictor of outcome for patients with gastric carcinoma. Ann Surg 235: 499-506, 2002.
9. Oyama K, Terashima M, Takagane A, Maesawa C, *et al*: Prognostic significance of peritoneal minimal residual disease in gastric cancer detected by reverse transcription-polymerase chain reaction. Br J Surg 91: 435-443, 2004.
10. Zeimet AG, Widschwendter M, Knabbe C, *et al*: Ascitic interleukin-12 is an independent prognostic factor in ovarian cancer. J Clin Oncol 16: 1861-1868, 1998.
11. Majima T, Ichikura T, Seki S, Takayama E, Hiraide H and Mochizuki H: Interleukin-10 and interferon-gamma levels within the peritoneal cavity of patients with gastric cancer. J Surg Oncol 78: 124-130, 2001.

12. Zhang B, Rong G, Wei H, *et al*: The prevalence of Th17 cells in patients with gastric cancer. Biochem Biophys Res Commun 374: 533-537, 2008.
13. Maruyama T, Kono K, Mizukami Y, *et al*: Distribution of Th17 cells and FoxP3(+) regulatory T cells in tumor-infiltrating lymphocytes, tumor-draining lymph nodes and peripheral blood lymphocytes in patients with gastric cancer. Cancer Sci 101: 1947-1954, 2010.
14. Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J and Wang RF: Generation and regulation of human $CD4^+$ IL-17-producing T cells in ovarian cancer. Proc Natl Acad Sci USA 105: 15505-15510, 2008.
15. Langowski JL, Zhang X, Wu L, *et al*: IL-23 promotes tumour incidence and growth. Nature 442: 461-465, 2006.
16. Kato T, Furumoto H, Ogura T, *et al*: Expression of IL-17 mRNA in ovarian cancer. Biochem Biophys Res Commun 282: 735-738, 2001.
17. Sfanos KS, Bruno TC, Maris CH, *et al*: Phenotypic analysis of prostate-infiltrating lymphocytes reveals T_H17 and T_{reg} skewing. Clin Cancer Res 14: 3254-3261, 2008.
18. Numasaki M, Fukushi J, Ono M, *et al*: Interleukin-17 promotes angiogenesis and tumor growth. Blood 101: 2620-2627, 2003.
19. Benchetrit F, Ciree A, Vives V, *et al*: Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. Blood 99: 2114-2121, 2002.
20. Zou W and Restifo NP: T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol 10: 248-256, 2010.
21. Iida T, Iwahashi M, Katsuda M, *et al*: Tumor-infiltrating $CD4^+$ Th17 cells produce IL-17 in tumor microenvironment and promote tumor progression in human gastric cancer. Oncol Rep 25: 1271-1277, 2011.
22. Kryczek I, Wei S, Zou L, *et al*: Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. J Immunol 178: 6730-6733, 2007.
23. Lissoni P, Mandalà M, Curigliano G, *et al*: Progress report on the palliative therapy of 100 patients with neoplastic effusions by intracavitary low-dose interleukin-2. Oncology 60: 308-312, 2001.
24. Lenzi R, Rosenblum M, Verschraegen C, *et al*: Phase I study of intraperitoneal recombinant human interleukin 12 in patients with Müllerian carcinoma, gastrointestinal primary malignancies, and mesothelioma. Clin Cancer Res 8: 3686-3695, 2002.
25. Fu QG, Meng FD, Shen XD and Guo RX: Efficacy of intraperitoneal thermochemotherapy and immunotherapy in intraperitoneal recurrence after gastrointestinal cancer resection. World J Gastroenterol 8: 1019-1022, 2002.
26. Ye ZJ, Zhou Q, Gu YY, *et al*: Generation and differentiation of IL-17-producing $CD4^+$ T cells in malignant pleural effusion. J Immunol 185: 6348-6354, 2010.
27. Derhovanessian E, Adams V, Hähnel K, *et al*: Pretreatment frequency of circulating IL-17^+ $CD4^+$ T-cells, but not Tregs, correlates with clinical response to whole-cell vaccination in prostate cancer patients. Int J Cancer 125: 1372-1379, 2009.
28. Kryczek I, Banerjee M, Cheng P, *et al*: Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. Blood 114: 1141-1149, 2009.
29. Zhang JP, Yan J, Xu J, *et al*: Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. J Hepatol 50: 980-989, 2009.
30. Liu J, Duan Y, Cheng X, *et al*: IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. Biochem Biophys Res Commun 407: 348-354, 2011.
31. Zhuang Y, Peng LS, Zhao YL, Shi Y, Mao XH, *et al*: $CD8^+$ T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer. Gastroenterology 143: 951.e8-962.e8, 2012.

Surg Today (2014) 44:1708–1715
DOI 10.1007/s00595-013-0782-3

ORIGINAL ARTICLE

Clinical benefits of thoracoscopic esophagectomy in the prone position for esophageal cancer

Makoto Iwahashi · Mikihito Nakamori · Masaki Nakamura · Toshiyasu Ojima · Masahiro Katsuda · Takeshi Iida · Keiji Hayata · Hiroki Yamaue

Received: 11 August 2013 / Accepted: 11 October 2013 / Published online: 20 November 2013

Abstract
Purposes The clinical benefits of thoracoscopic radical esophagectomy in the prone position compared to conventional open esophagectomy have not been fully documented.
Methods Forty-six patients with esophageal cancer who underwent MIE in the prone position (MIE-P group) were enrolled, and 46 case-matched controls that underwent open esophagectomy (OE group) were identified using propensity score methods to achieve a valid comparison of outcomes between MIE and open esophagectomy.
Results The duration of systemic inflammatory response syndrome was shorter in the MIE-P group than in OE group ($P = 0.005$). The time to first walking was earlier in the MIE-P group ($P < 0.001$). Although the vital capacity ratio (%VC) declined after the operation in both groups, the change ratio of the %VC was 85.3 % in the MIE-P group and 69.6 % in the OE group ($P < 0.001$). No mortality occurred in either group. The postoperative morbidity rate was lower in the MIE-P group (13 %) than in the OE group (30.4 %) ($P = 0.020$). Two patients (4.3 %) in the OE group and one patient in the MIE-P group (2.2 %) had pneumonia.
Conclusions MIE in the prone position was associated with less impairment of the pulmonary function, earlier recovery of activity and lower subsequent morbidity compared to open esophagectomy. Further investigation of the long-term outcomes is, therefore, needed.

M. Iwahashi · M. Nakamori · M. Nakamura · T. Ojima · M. Katsuda · T. Iida · K. Hayata · H. Yamaue (✉)
Second Department of Surgery, School of Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8510, Japan
e-mail: yamaue-h@wakayama-med.ac.jp

Keywords Minimally invasive esophagectomy · Thoracoscopic esophagectomy · Esophageal cancer · Prone position · Postoperative pulmonary function · Postoperative morbidity

Introduction

A number of studies have demonstrated the safety and possible advantages of minimally invasive esophagectomy (MIE) in selected cohorts of patients [1–6]. MIE is, therefore, being performed with increasing frequency [7], and evidence of the short-term benefits of MIE over traditional open procedures with a similar oncological outcome is accumulating. Most comparative studies have demonstrated clinical advantages of MIE, such as less blood loss, a shorter intensive care unit (ICU) stay and similar survival. Nevertheless, systemic reviews of studies involving MIE have been equivocal and have failed to draw definitive conclusions [8]. A population-based national study in England has shown that there were no significant benefits demonstrated in the mortality and overall morbidity [9]. Most recently, a randomized controlled trial has shown the benefits of MIE in terms of a lower incidence of pulmonary infection and better quality of life compared to open esophagectomy [10].

Various types of MIE for patients with esophageal cancer have been described, and the most generally performed technique involves thoracoscopic mobilization of the esophagus in the left lateral decubitus position [1, 2, 4, 11]. Recently, the advantages of thoracoscopic esophageal mobilization in the prone position have also been reported [12, 13]. Compared to the left lateral decubitus position, the prone position allows better operative exposure and improved surgeon ergonomics, resulting in reduced

pulmonary complications, a shorter operation and less blood loss [12, 13]. The advantages in terms of surgeon ergonomics and operative exposure were apparent even in an aggressive esophagectomy with a three-field lymphadenectomy [14]. Therefore, thoracoscopic esophagectomy in the prone position would be expected to have potential benefits in radical esophagectomy with extended lymph node dissection for patients with esophageal cancer. However, the efficacy of this approach as a minimally invasive surgery compared to conventional open esophagectomy has not yet been fully documented [15].

The aim of the present study was to examine the clinical benefits of the thoracoscopic radical esophagectomy with extensive lymphadenectomy in the prone position as a minimally invasive surgery compared to open esophagectomy for patients with esophageal cancer, using a propensity score-matching analysis to evaluate the outcomes without selection bias.

Patients and methods

Patient populations

From January 2004 to December 2011, 288 patients with esophageal cancer underwent esophagectomy at Wakayama Medical University Hospital (WMUH). Until 2009, our traditional standard surgical procedure for patients with esophageal cancer was transthoracic open esophagectomy. Thoracoscopic esophagectomy with extensive lymphadenectomy in the prone position was adopted in 2009 and was performed in 51 patients with thoracic or abdominal esophageal cancer at WMUH. The selection criteria for this minimally invasive procedure were as follows: no previous thoracic surgery, no possibility of severe pleural adhesion and no previous radiation therapy to the esophagus. During the first year, this procedure was only performed in patients with clinical T1 tumors, but from the second year, it was also performed in patients with clinical T2 or T3 tumors. From among these 51 patients, 46 patients who underwent curative resection with gastric conduit reconstruction were enrolled in our study (MIE-P group). A case-matched control group (OE group) was identified from patients who underwent open transthoracic esophagectomy between January 2004 to December 2011 using propensity score methods to achieve a valid comparison of outcomes between MIE and open esophagectomy.

The patients were staged according to the TNM classification (7th edition) of the American Joint Committee on Cancer and the International Union Against Cancer.

This study was approved by the Ethics Committee on Human Research of WMUH, and informed consent was obtained from all patients.

Perioperative management

The perioperative care and anesthesia of esophageal cancer surgery are standardized at WMUH, as reported previously [16]. Respiratory physiotherapy and oral care were performed in all patients in our study groups. Intravenous methylprednisolone (125 mg) was administered twice to each patient, at the beginning of the thoracic procedure and at the end of the surgery. The patients were usually admitted to the ICU immediately after the operation. The patients were discharged from the ICU after extubation if their conditions remained stable. Epidural analgesia was used routinely for postoperative pain management for 1 week. Postoperative physical rehabilitation strategies, such as deep breathing with huffing and coughing and postural drainage to assist breathing and expectoration, were performed in all patients. In addition, the patients were instructed to stand up and walk as early as possible.

Surgical procedures

The patients underwent a radical esophagectomy with a total mediastinal lymphadenectomy (extended two-field) or three-field lymph node dissection via a cervicothoracoabdominal approach. The operation was conducted in three stages. In the first stage, the intrathoracic manipulation was performed as described in detail in the following sections. The patient was placed in the prone position for the MIE procedure or in the left lateral decubitus position for the open esophagectomy. In the second stage, the patient was rotated to a supine position, and gastric mobilization and abdominal lymphadenectomy (around the left gastric pedicle and celiac axis) were performed. The entire isolated thoracic esophageal specimen with dissected lymph nodes (LNs) was removed through the esophageal hiatus, and a gastric conduit was constructed. In the MIE-P group, a hand-assisted laparoscopic approach was used for these abdominal procedures. However, eight of 46 patients were converted to open surgery because of obvious LN metastases around the celiac axis or due to abdominal adhesion from previous upper abdominal surgery. In the third stage, the cervical esophagus was mobilized, and a cervical lymphadenectomy was performed. Finally, a gastric conduit was delivered up through the retrosternal rout or through the posterior mediastinum to reconstruct the anastomosis with the cervical esophagus in the cervical field.

Thoracoscopic esophagectomy in the prone position

The patient was placed in the prone position while under epidural and general anesthesia, which used a single-lumen flexible endotracheal tube with a blocking balloon

inserted into the right main bronchus for single-lung ventilation. The port positions were as follows: A 12-mm blunt port was first carefully inserted into the seventh intercostal space (ICS) behind the posterior axillary line, and CO_2 was then insufflated at a pressure of 6 mmHg. Four other ports were inserted under thoracoscopic control: a 12-mm port in the ninth ICS on the scapular angle line for the thoracoscope, a 5-mm port in the fifth ICS on the midaxillary line, a 12-mm port in the third ICS on the midaxillary line and a 5-mm port in the seventh ICS on the scapular angle line. Intrathoracic procedures began with mobilizing the middle and lower esophagus with regional LNs along the layer that exposed the pericardium, the descending aorta and the left mediastinal pleura. The azygos vein was then divided and the right bronchial artery was also divided. The entire thoracic duct was carefully preserved. Then, the upper thoracic esophagus, the right main branch of the vagal nerve and the right subclavian artery were exposed. The LNs around the right recurrent nerve were dissected up to the thyroid gland. Then, the upper thoracic esophagus was circumferentially mobilized, and tape was placed around the esophagus to facilitate retraction. The upper thoracic esophagus was retracted to the dorsal side by pulling the tape via thread outside the thorax, and the trachea was rolled back to the right and ventrally by a grasper holding small gauze, as described previously [14]. The tissue, including the LNs around the left recurrent nerve, was precisely dissected. Next, the esophagus was divided, the LNs below the aortic arch were dissected and the bilateral esophageal branches of the vagal nerve were divided, while pulmonary branches were preserved. The subcarinal LNs were dissected. The thoracic esophagus was completely mobilized circumferentially, and the paraesophageal LNs were dissected and maintained *en bloc* with the surgical specimen. A single 28-F chest tube was placed through the 12-mm port site for postoperative drainage.

Open transthoracic esophagectomy

The patient was placed in the left lateral decubitus position. A right vertical incision (12 cm) was made, and thoracotomy was performed through the fourth ICS. A 12-mm port was placed in the sixth ICS on the midaxillary line for the thoracoscope. The operator looked directly at the surgical field, and assistant surgeons viewed the area through a video monitor. The mobilization of the esophagus and lymphadenectomy was performed almost the same way as in the thoracoscopic esophagectomy. However, the definite difference from thoracoscopic procedures was that the retraction of the lung, the trachea, the right bronchus and the heart by assistants was necessary to expose the surgical field.

Patient monitoring and data collection

The data were recorded prospectively for all patients who underwent thoracoscopic esophagectomy in the prone position. However, the data were retrospectively collected from medical records for patients in the control OE group. Patients with disorders such as angina pectoris or previous myocardial infarction were defined as having cardiovascular disease. Patients with abnormal pulmonary function on spirograms (vital capacity ratio [%VC] <70 % or forced expiratory volume in 1 s [FEV_1]/forced vital capacity [FVC] <60 %) were defined as having comorbid pulmonary disease. Diabetes mellitus was noted if the patient had a fasting blood glucose concentration >126 mg/dL or was receiving antidiabetic therapy. Patients with chronic hepatitis or liver cirrhosis that required treatment were defined as having liver disease. Patients with renal disease that required treatment were defined as having renal disease.

Evaluation of the postoperative clinical course and respiratory function

The duration of systemic inflammatory response syndrome (SIRS) and ICU stay, the time to first independent sitting, time to first standing and time to first walking outside of the room were evaluated by comparing the two groups. The white blood cell (WBC) count and C-reactive protein (CRP) level were compared before the operation, and on postoperative days (PODs) 1, 3 and 5. Postoperative complications were analyzed according to the Clavien–Dindo classification [17], and postoperative complications greater than grade II were regarded as being clinically significant. The surgical mortality (Clavien–Dindo classification; grade V) included in-hospital deaths (by POD 90). Pulmonary function was evaluated by the %VC and the ratio of FEV1 to FVC (FEV1%) in spirograms before and 3 to 4 weeks after the operation. The change ratio of the %VC (postoperative %VC/preoperative %VC × 100) and the change ratio of the FEV1% (postoperative FEV1%/preoperative FEV1% × 100) were also compared between the two groups.

Statistical analysis

The case-matched control group was identified using a propensity score matching method. The propensity score was calculated for each patient by a logistic regression analysis based on the following variables: age, sex, tumor location, depth of tumor invasion, the degree of LN involvement, pathological stage, histological type of tumor, preoperative chemotherapy and concomitant diseases. The quantitative results were expressed as the mean ± standard

Table 1 Patient and tumor demographics

	OE ($n = 46$)	MIE-P ($n = 46$)	P value[§]
Age[a]	65.9 ± 8.9	65.0 ± 10.2	0.689
Sex			0.797
Male/female	37/9	36/10	
Location of tumor			0.663
Upper	4	2	
Middle	30	30	
Lower	12	14	
Depth of tumor invasion			1.00
T1	35	35	
T2	4	4	
T3	7	7	
Lymph node metastases			0.717
N0	27	28	
N1	8	8	
N2	5	7	
N3	6	3	
Stage			0.947
IA + IB	25	26	
IIA + IIB	13	14	
IIIA + IIIB + IIIC	5	4	
IV	3	2	
Histology			1.00
Squamous cell carcinoma	44	44	
Adenocarcinoma	1	1	
Others	1	1	
Neoadjuvant chemotherapy			0.216
Yes/no	4/42	8/38	
Lymph node dissection			
Extended 2-field[b]/3-field	35/11	38/8	0.440
Comorbidity			
Cardiac Yes/no	2/44	1/45	0.500
Respiratory Yes/no	8/38	9/37	0.788
Diabetes Yes/no	6/40	2/44	0.133
Liver Yes/no	3/43	2/44	0.500

OE open esophagectomy, *MIE-P* minimally invasive esophagectomy in the prone position

§ P value between the OE group and the MIE-P group

[a] Data are expressed as the mean ± SD

[b] Extended 2-field: total mediastinal lymphadenectomy

deviation or the medians (range). The statistical analysis was performed by Student's t test, the Mann–Whitney U test or Fisher's exact test, as appropriate. Values of $P < 0.05$ were considered significant. All statistical analyses were carried out using the SPSS software package (v. 19.0; SPSS, Chicago, IL).

Table 2 Procedure-related data

	OE ($n = 46$)	MIE-P ($n = 46$)	P value[‡]
Length of operation (min)[a]			
Total	488 ± 59	609 ± 54	<0.001
Chest	234 ± 44	362 ± 40	<0.001
Estimated blood loss (mL)[b]	255 (72–925)	125 (30–420)	<0.001
Number of dissected lymph nodes[b]			
Chest	22 (9–54)	23 (9–36)	0.774
Along the recurrent nerve	6 (1–14)	7 (1–17)	0.253
Length of ICU stay (d)[c]	1.07 (1–3)	1.07 (1–2)	0.669
Duration of SIRS (d)[c]	0.82 (0–4)	0.22 (0–3)	0.005
Days until independent sitting (d)[c]	1.74 (1–5)	1.02 (1–2)	<0.001
Days until first standing (d)[c]	1.82 (1–5)	1.02 (1–2)	<0.001
Days until first walking (d)[c]	3.47 (1–21)	1.02 (1–2)	<0.001

OE open esophagectomy, *MIE-P* minimally invasive esophagectomy in the prone position, *SIRS* systemic inflammatory response syndrome

‡ P value between the OE group and the MIE-P group

[a] Data are expressed as the mean ± SD

[b] Data are expressed as the medians (range)

[c] Data are expressed as the means (range)

Results

Patients and tumor demographics

Forty-six patients who underwent thoracoscopic esophagectomy in the prone position were enrolled in this study and were matched 1:1 with 46 patients who underwent open esophagectomy. The patient and tumor demographics and the associated preoperative comorbidities for the matched groups are summarized in Table 1. There were no significant differences between the groups.

Surgical outcomes

The surgical outcomes after esophagectomy are shown in Table 2. The mean total length of the operation was significantly longer in the MIE-P group (609 ± 54 min) than in the OE group (488 ± 59 min), which was due to the significantly longer duration of the thoracoscopic procedure ($P < 0.001$). The median estimated blood loss in the MIE-P group was 125 mL (range 30–420 mL) and that in the OE group was 255 mL (range 72–925 mL); the difference was statistically significant between the two groups ($P \leq 0.001$). The number of dissected LNs in the chest and along the recurrent nerve in the OE group was similar to that in the MIE-P group. There were no significant differences in the length of ICU stay between

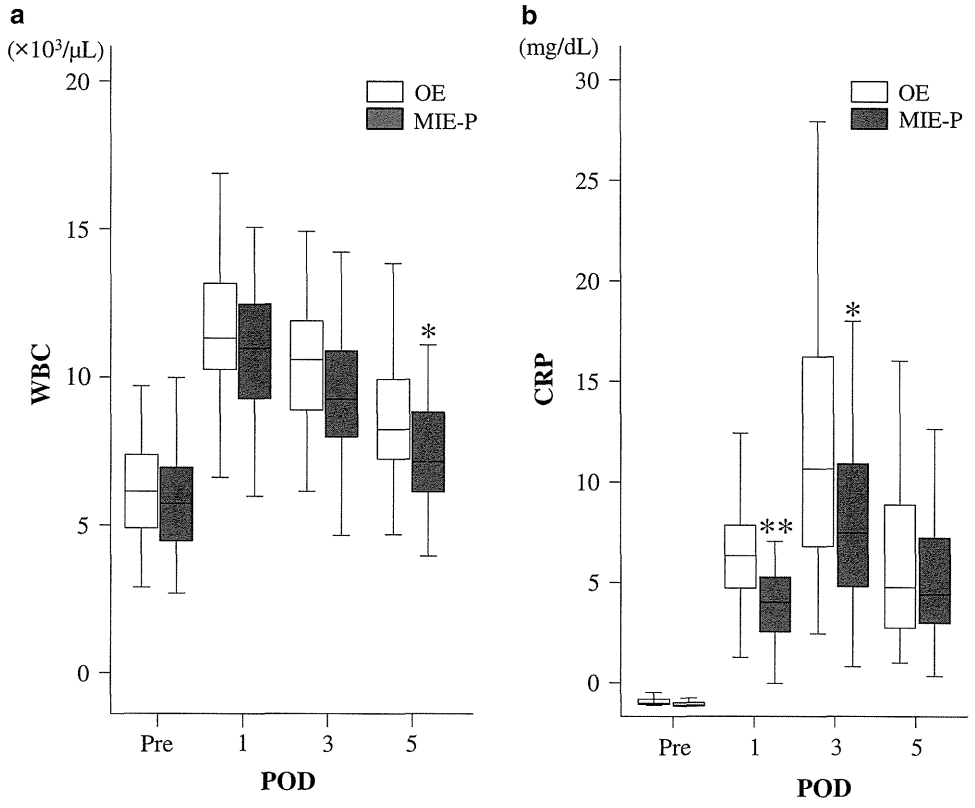

Fig. 1 The pre- and postoperative changes in the white blood cell (WBC) count (**a**) and serum C-reactive protein (CRP) level (**b**). *The horizontal bars* indicate the median values. *The vertical bars* indicate the range, and the horizontal boundaries of the *boxes* represent the first and third quartiles. *OE* open esophagectomy, *MIE-P* minimally invasive esophagectomy in the prone position. $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different between the OE group and the MIE-P group

the two groups, while the duration of SIRS was shorter in the MIE-P group than in the OE group ($P = 0.005$). In terms of the activity of daily living (ADL), the time to first independent sitting, time to first standing and time to first walking outside of the room were all significantly earlier in the MIE-P group compared to the OE group ($P < 0.001$).

The perioperative changes in the WBC count and serum CRP level are shown in Fig. 1. The WBC count peaked on POD 1, and the CRP level peaked on POD 3. The peak WBC counts were similar between the OE and MIE-P groups; however, the WBC count in the MIE-P group was significantly lower than that in the OE group on POD 5 ($P < 0.05$). The serum CRP levels in the MIE-P group were significantly lower than those in the OE group on POD 1 and 3 ($P < 0.01$, $P < 0.05$).

Perioperative pulmonary function

The perioperative changes in the respiratory function were evaluated by spirography (Table 3). The %VC was decreased after the operation in both the OE and MIE-P groups. Importantly, the %VC was significantly higher in the MIE-P group than in the OE group after the operation ($P = 0.002$), although it was similar between the two groups before the operation. The change ratio of the %VC was significantly different between the two groups ($P < 0.001$): 85.3 % in the MIE-P group and 69.6 % in the OE group. The FEV1% was not influenced by the operation in either group. There were no differences in the FEV1% between the two groups before or after the operation.

Table 3 Perioperative changes in the pulmonary functions

	OE ($n = 46$)	MIE-P ($n = 46$)	P value[‡]
%VC			
Preoperative	112.8 ± 17.6	108.9 ± 15.7	0.412
Postoperative	78.0 ± 12.9	92.6 ± 16.3	0.002
Change ratio[a]	69.6 ± 8.8	85.3 ± 10.7	<0.001
FEV1%			
Preoperative	74.8 ± 10.4	76.4 ± 9.7	0.558
Postoperative	77.9 ± 9.5	79.0 ± 9.5	0.683
Change ratio[b]	104.8 ± 9.5	103.9 ± 9.5	0.687

Data are expressed as the mean ± SD

OE open esophagectomy, *MIE-P* minimally invasive esophagectomy in the prone position, *%VC* %vital capacity, *FEV1%* the ratio of the forced expiratory volume in one second (FEV_1) to the forced vital capacity (FVC)

[‡] P value between the OE group and the MIE-P group

[a] Change ratio = postoperative %VC/preoperative %VC × 100

[b] Change ratio = postoperative FEV1%/preoperative FEV1% × 100

Table 4 Postoperative morbidity and mortality

	Number (%)		
	OE ($n = 46$)	MIE-P ($n = 46$)	P value‡
Mortality	0	0	1.0
Complications[a]	14 (30.4)	6[b] (13.0)	0.020
Pneumonia	2 (4.3)	1 (2.2)	0.500
Grade II/IIIa/IIIb, IV	1/1/0	0/1/0	
Chylothorax	1 (2.2)	0	0.315
Grade II/IIIa/IIIb, IV	1/0/0	–	
Anastomotic leakage	4 (8.7)	1 (2.2)	0.181
Grade II/IIIa/IIIb, IV	3/1/0	0/1/0	
Surgical site infection	2 (4.3)	0	0.153
Grade II/IIIa/IIIb, IV	2/0/0	–	
Recurrent nerve palsy	5 (10.9)	5 (10.9)	1.0
Grade II/IIIa/IIIb, IV	4/1/0	4/1/0	

‡ P value between the OE group and the MIE-P group

OE open esophagectomy, *MIE-P* minimally invasive esophagectomy in the prone position

[a] Clavien–Dindo classification

[b] One of six patients developed both pneumonia and anastomotic leakage

Postoperative morbidity and mortality

There was no operative mortality associated with either approach in this study. The incidence of postoperative complications was significantly lower in the MIE-P group (13 %) than in the OE group (30.4 %) ($P = 0.020$). The specific complications of grades II, III and IV according to the Clavien–Dindo classification are summarized in Table 4. None of the patients developed severe complications of grade IIIb or IV. Two patients (4.3 %) in the OE group and one patient in the MIE-P group (2.2 %) had pneumonia. Four patients (8.7 %) in the OE group and one (2.2 %) in the MIE-P group developed anastomotic leakage. One patient (2.2 %) developed chylothorax and two patients (4.3 %) developed a surgical site infection in the OE group, while no patients in the MIE-P group developed chylothorax or a surgical site infection. Five patients (10.9 %) in each group, respectively, developed recurrent nerve palsy. There were no statistically significant differences in the incidence of each specific complication between the two groups. The statistical data are shown in Table 4.

Discussion

The present study shows that MIE in the prone position has several advantages over conventional open esophagectomy in the radical surgical treatment with extensive lymphadenectomy. The patients who underwent MIE had an earlier recovery of the ADL, lower levels of the inflammatory response, less impairment of pulmonary function and lower morbidity. In addition, the propensity score matching technique was used to minimize any selection bias, and the patient and tumor demographics showed good matching.

The minimally invasive procedure in itself is expected to reduce the surgical stress after esophagectomy. However, there have been few studies that have evaluated the postoperative inflammatory response in patients who underwent MIE. The most recent study demonstrated that the serum levels of inflammatory cytokines, such as interleukin-6, immediately after the operation are significantly lower in patients who underwent MIE in the prone position than in those who underwent conventional open esophagectomy; further, the incidence of SIRS was lower in patients who underwent MIE [18]. In the present study, the peak serum levels of CRP in the MIE-P group were significantly lower than those in the OE group, and the duration of SIRS was significantly shorter in the MIE-P group than in the OE group. This is consistent with the study by Tsujimoto et al. [18], and it suggests that MIE in the prone position in itself is a less invasive procedure.

Less invasive surgical procedures may allow patients to achieve comparatively earlier recovery. A thoracoscopic esophagectomy minimizes the injury to the chest wall and would, therefore, contribute to an early physical recovery. In this context, there have not been enough reports that have examined the postoperative course of recovery. In the present study, the time to first independent sitting, time to first standing and time to first walking outside of the room were significantly earlier in the MIE-P group than in the OE group. All patients except one in the MIE-P group could walk outside of their room on POD 1. However, there is a possibility of some bias in the background of the patients because the early recovery program was launched in 2007 at our hospital.

The postoperative pulmonary function may affect not only the postoperative morbidity, but also the postoperative quality of life. In particular, the vital capacity is usually impaired after open transthoracic surgery due to the injury to the chest wall, including respiratory muscles. Preservation of the pulmonary function is one of the most important issues after esophagectomy. However, this outcome has not been fully investigated in previous studies, especially during the early postoperative period. In the present study, there were no significant differences in the FEV1% between the OE and MIE-P groups before or after the operation. This is likely because the airway resistance is not influenced by esophagectomy, even when the open approach is used. In contrast, the %VC was diminished 3 to 4 weeks after the operation in both groups. Importantly, however, the impairment of the %VC was significantly lower in the MIE-P group than in the OE group after the

operation. MIE can minimize the injury to the chest wall, and moreover, especially when performed in the prone position, it may minimize the direct damage to the lung. This is because the physical retraction of the lung is not needed in the prone position. Therefore, the vital capacity could be preserved even in the early postoperative period following MIE. These results suggest that MIE in the prone position has an obvious benefit in terms of the preservation of pulmonary function.

The postoperative morbidity and mortality remain important issues in patients who undergo esophagectomy for esophageal cancer. Considering the various advantages of minimally invasive surgical procedures, MIE has been expected to reduce the morbidity and mortality. Although MIE is reported to be an independent factor predicting a lower frequency of postoperative respiratory failure [19], many previous reports have failed to demonstrate that MIE actually reduces the morbidity and mortality [1, 2, 4, 8]. In addition, a recent population-based national study from England showed that there were no significant benefits demonstrated in the mortality and mobility [9]. With regard to MIE in the prone position, there have been no studies that have demonstrated obvious advantages over conventional open esophagectomy in terms of postoperative mortality and morbidity [5, 6, 12]. This lack of evidence may be due to a selection bias of patients, as well as the considerable variations in the definition of postoperative complications. In the present study, we used the propensity score matching technique to diminish the selection bias, and as a matter of course, we analyzed the postoperative complications according to the Clavien–Dindo classification [17].

In the present study there was no mortality due to either approach, and the overall morbidity was generally low compared to previous reports [1, 3–5, 12, 19]. The morbidity rate was significantly lower in the MIE-P group (13.0 %) than in the OE group (30.4 %). These results suggest that thoracoscopic esophagectomy with extensive lymphadenectomy in the prone position is less invasive compared to conventional open esophagectomy. The incidence of pneumonia was 2.2 % in the ME-P group and 4.3 % in the OE group. Both rates were low compared with other reports [1, 3–5, 9, 12], and no statistically significant difference could be detected between the groups in terms of the development of pneumonia.

In conclusion, we examined the clinical benefits of thoracoscopic esophagectomy in the prone position for patients with esophageal cancer compared to propensity score-matched control patients who underwent open esophagectomy for esophageal cancer. MIE with extensive lymphadenectomy in the prone position had obvious advantages over conventional open esophagectomy in terms of the lower levels of inflammatory response, less impairment of the pulmonary function, earlier recovery of the ADL and lower subsequent morbidity. However, the present study has some limitations with regard to showing the definitive benefits of MIE in the prone position because it was a nonrandomized study. Further investigations are also needed to determine the long-term outcomes of this procedure, including the survival.

Conflict of interest Makoto Iwahashi and the co-authors have no conflicts of interest to declare.

References

1. Luketich JD, Alvelo-Rivera M, Buenaventura PO, Christie NA, McCaughan JS, Litle VR, et al. Minimally invasive esophagectomy: outcomes in 222 patients. Ann Surg. 2003;238:486–94.
2. Nguyen NT, Follette DM, Wolfe BM, Schneider PD, Roberts P, Goodnight JE Jr. Comparison of minimally invasive esophagectomy with transthoracic and transhiatal esophagectomy. Arch Surg. 2000;135:920–5.
3. Nguyen NT, Hinojosa MW, Smith BR, Chang KJ, Gray J, Hoyt D. Minimally invasive esophagectomy: lessons learned from 104 operations. Ann Surg. 2008;248:1081–91.
4. Osugi H, Takemura M, Higashino M, Takada N, Lee S, Kinoshita H. A comparison of video-assisted thoracoscopic oesophagectomy and radical lymph node dissection for squamous cell cancer of the oesophagus with open operation. Br J Surg. 2003;90:108–13.
5. Smithers BM, Gotley DC, Martin I, Thomas JM. Comparison of the outcomes between open and minimally invasive esophagectomy. Ann Surg. 2007;245:232–40.
6. Zingg U, McQuinn A, DiValentino D, Esterman AJ, Bessell JR, Thompson SK, et al. Minimally invasive versus open esophagectomy for patients with esophageal cancer. Ann Thorac Surg. 2009;87:911–9.
7. Lazzarino AI, Nagpal K, Bottle A, Faiz O, Moorthy K, Aylin P. Open versus minimally invasive esophagectomy: trends of utilization and associated outcomes in England. Ann Surg. 2010;252:292–8.
8. Decker G, Coosemans W, De Leyn P, Decaluwe H, Nafteux P, Van Raemdonck D, et al. Minimally invasive esophagectomy for cancer. Eur J Cardiothorac Surg. 2009;35:13–20.
9. Mamidanna R, Bottle A, Aylin P, Faiz O, Hanna GB. Short-term outcomes following open versus minimally invasive esophagectomy for cancer in England: a population-based national study. Ann Surg. 2012;255:197–203.
10. Biere SSAY, van Berge Henegouwen MI, Maas KW, Bonavina L, Rosman C, Garcia JR, et al. Minimally invasive versus open oesophagectomy for patients with oesophageal cancer: a multicentre, open-label, randomised controlled trial. Lancet. 2012;379:1887–92.
11. Akaishi T, Kaneda I, Higuchi N, Kuriya Y, Kuramoto J, Toyoda T, et al. Thoracoscopic en bloc total esophagectomy with radical mediastinal lymphadenectomy. J Thorac Cardiovasc Surg. 1996;112:1533–40.
12. Fabian T, Martin J, Katigbak M, McKelvey AA, Federico JA. Thoracoscopic esophageal mobilization during minimally invasive esophagectomy: a head-to-head comparison of prone versus decubitus positions. Surg Endosc. 2008;22:2485–91.
13. Palanivelu C, Prakash A, Senthilkumar R, Senthilnathan P, Parthasarathi R, Rajan PS, et al. Minimally invasive esophagectomy: thoracoscopic mobilization of the esophagus and mediastinal lymphadenectomy in prone position—experience of 130 patients. J Am Coll Surg. 2006;203:7–16.

14. Noshiro H, Iwasaki H, Kobayashi K, Uchiyama A, Miyasaka Y, Masatsugu T, et al. Lymphadenectomy along the left recurrent laryngeal nerve by a minimally invasive esophagectomy in the prone position for thoracic esophageal cancer. Surg Endosc. 2010;24:2965–73.
15. Watanabe M, Baba Y, Nagai Y, Baba H. Minimally invasive esophagectomy for esophageal cancer: an updated review. Surg Today. 2013;43:237–44.
16. Nakamura M, Iwahashi M, Nakamori M, Ishida K, Naka T, Iida T, et al. An analysis of the factors contributing to a reduction in the incidence of pulmonary complications following an esophagectomy for esophageal cancer. Langenbecks Arch Surg. 2008;393:127–33.
17. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg. 2004;240:205–13.
18. Tsujimoto H, Takahata R, Nomura S, Yaguchi Y, Kumano I, Matsumoto Y, et al. Video-assisted thoracoscopic surgery for esophageal cancer attenuates postoperative systemic responses and pulmonary complications. Surgery. 2012;151:667–73.
19. Zingg U, Smithers BM, Gotley DC, Smith G, Aly A, Clough A, et al. Factors associated with postoperative pulmonary morbidity after esophagectomy for cancer. Ann Surg Oncol. 2011;18:1460–8.

IJC
International Journal of Cancer

Antitumor immune response of dendritic cells (DCs) expressing tumor-associated antigens derived from induced pluripotent stem cells: In comparison to bone marrow-derived DCs

Hiromitsu Iwamoto, Toshiyasu Ojima, Keiji Hayata, Masahiro Katsuda, Motoki Miyazawa, Takeshi Iida, Masaki Nakamura, Mikihito Nakamori, Makoto Iwahashi and Hiroki Yamaue

Second Department of Surgery, Wakayama Medical University, School of Medicine, Wakayama 641-8510, Japan

It is generally accepted that the difficulty in obtaining a sufficient number of functional dendritic cells (DCs) is a serious problem in DC-based immunotherapy. Therefore, we used the induced pluripotent stem (iPS) cell-derived DCs (iPSDCs). If the therapeutic efficacy of iPSDCs is equivalent to that of bone marrow-derived DCs (BMDCs), then the aforementioned problems may be solved. In our study, we induced iPSDCs from iPS cells and examined the capacity for maturation of iPSDCs compared to that of BMDCs in addition to the capacity for migration of iPSDCs to regional lymph nodes. We adenovirally transduced the hgp100 gene, natural tumor antigens, into DCs and immunized mice once with the genetically modified DCs. The cytotoxic activity of CD8 (+) cytotoxic T lymphocytes (CTLs) was assayed using a ^{51}Cr-release assay. The therapeutic efficacy of the vaccination was examined in a subcutaneous tumor model. Our results showed that iPSDCs have an equal capacity to BMDCs in terms of maturation and migration. Furthermore, hgp100-specific CTLs were generated in mice immunized with genetically modified iPSDCs. These CTLs exhibited as high a level of cytotoxicity against B16 cells as BMDCs. Moreover, vaccination with the genetically modified iPSDCs achieved as high a level of therapeutic efficacy as vaccination with BMDCs. Our study clarified experimentally that genetically modified iPSDCs have an equal capacity to BMDCs in terms of tumor-associated antigen-specific therapeutic antitumor immunity. This vaccination strategy may therefore be useful for future clinical application as a cancer vaccine.

Tumor Immunology

Dendritic cells (DCs) are potent antigen-presenting cells that play a critical role in the initiation of antitumor immune responses.[1–3] Many cancer patients worldwide have been treated with cancer vaccine therapy using DCs. Our previous Phase I clinical trial of cancer vaccine therapy using carcinoembryonic antigen peptide-pulsed DCs found the clinical effects of this therapy to be insufficient.[4] Therefore, we next used a gene-based vaccination strategy that used DCs adenovirally transduced with the entire tumor-associated antigen (TAA) gene. We demonstrated that DCs adenovirally transduced with the TAA gene are effective in inducing TAA-specific cytotoxic T lymphocytes (CTLs) and that these cells elicit potent antitumor responses, especially in gastrointestinal solid tumors.[5–8] These findings suggest that this strategy would be useful in clinical application as a cancer vaccine in patients with gastrointestinal tumors, and clinical trials evaluating such applications are now under consideration.

The issue of limited cells is a serious obstacle for DC vaccine therapy. DCs created for clinical use are generated from the peripheral blood monocytes of patients. Therefore, a large amount of blood must be collected or leukapheresis must be performed, which is both expensive and time-consuming. An additional problem is that the number of DCs is reduced in the peripheral blood and the function of DCs is impaired in cancer patients.[9,10] Therefore, obtaining a sufficient number of functional DCs remains a serious problem for the application of DC vaccine therapy.

Recent studies have revealed that embryonic stem cell-like pluripotent stem cells, known as induced pluripotent stem (iPS) cells, can be generated from murine and human fibroblasts.[11,12] Furthermore, it has been reported that DCs can be successfully derived from murine iPS cells (iPSDCs).[13] If the therapeutic efficacy of iPSDCs is equivalent to that of bone marrow-derived DCs (BMDCs), then the aforementioned problems may be solved. Therefore, in our study, we transduced the TAA gene into iPSDCs and examined whether the genetically modified iPSDCs can induce TAA-specific CTLs as effectively as BMDCs.

In our study, iPSDCs were adenovirally transduced with the entire natural tumor antigen hgp100 gene,[14] and whether vaccination with these genetically engineered iPSDCs can

Key words: iPSDCs, DCs vaccine therapy, TAA-specific, genetically modified DCs, antitumor immune response
Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; **Grant number:** 23791492
DOI: 10.1002/ijc.28367
History: Received 14 Feb 2013; Accepted 19 June 2013; Online 3 July 2013
Correspondence to: Toshiyasu Ojima, Second Department of Surgery, Wakayama Medical University, School of Medicine, 811-1 Kimiidera, Wakayama 641-8510, Japan, Tel.: +81-73-441-0613, Fax: +81-73-446-6566, E-mail: tojima@wakayama-med.ac.jp

What's new?
Dendritic cells (DCs) loaded with tumor antigens are important components of immune-based cancer therapies. However, the limited recovery of bone-marrow derived DCs from the blood of cancer patients is a serious obstacle to the common use of DC-based treatments. Here, the authors present an important alternative. They show that DCs derived from induced pluripotent stem cells (iPSCs) are equal in antigen-presentation and migration properties to bone-marrow derived DCs. In a model using adenoviral transduction of the gene encoding tumor-associated antigen, they demonstrate similar antitumor immune responses elicited by both DC types. This new technique may help overcome the limitations of traditional DC-based therapies and may represent a significant step towards a more effective personalized anticancer medicine.

induce strong therapeutic antitumor immunity equivalent to vaccination with genetically engineered BMDCs was assessed.

Material and Methods

Mice

Female C57BL/6 (H-2^b) mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. Six- to 8-week-old mice were used for the experiments. All animal experiments were performed in accordance with the Japanese Government's Animal Protection and Management Law (No. 105) and Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (No. 88), as well as the guidelines for animal experiments of Wakayama Medical University. Our study plan was approved by the Committee of Animal Experiments (No. 520) and the Committee of Gene Recombination (No. 23-5) of Wakayama Medical University.

Cell lines

The murine embryonic fibroblast-derived iPS cell line iPS-MEF-Ng-20D-17[15] was provided by the RIKEN BioResource Center (Ibaraki, Japan). The iPS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% embryonic stem screened fetal bovine serum (FBS) (Thermo Scientific, Yokohama, Japan), 2 mM L-glutamine (Thermo Scientific), 100 U/ml of penicillin, 100 mg/ml of streptomycin (Life Technologies Co., Carlsbad, CA), nonessential amino acids (Life Technologies) and 50 μM of 2-mercaptoethanol (2-ME) (Life Technologies) on feeder cell layers of mitomycin C-treated murine SNL76/7 cells (European Collection of Cell Cultures, London, UK).[16] Murine bone marrow stromal cells, OP9,[17] were provided by the RIKEN BioResource Center. The cells were maintained in α-MEM supplemented with 20% FBS and seeded onto gelatin-coated dishes before use as feeder cells. The C57BL/6-derived B16 melanoma cell lines expressing hgp100 were maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine. The murine chemically induced colon carcinoma cell line MC38 was maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 2 mM L-glutamine. The murine cell line derived from lymphoma, YAC-1, was purchased from RIKEN BioResource Center and maintained in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 2 mM L-glutamine. Recombinant murine (rm) tumor necrosis factor (TNF)-α and granulocyte macrophage colony stimulating factor (GM-CSF) were purchased from Peprotech (London, UK).

Recombinant adenoviral vectors

The adenoviral vector expressing hgp100, AxCAhgp100,[18] was provided by the RIKEN BioResource Center. The recombinant AxCALacz expressing a *Lacz* reporter gene was generated according to the COS-TPC method, as previously described.[5]

Generation of DCs from bone marrow cells

DCs were obtained from murine bone marrow precursors as previously described.[19] In brief, murine bone marrow cells (2.0×10^6) were cultured in 100-mm dishes in 10 ml of complete medium containing 200 units/ml of rmGM-CSF. The complete medium consisted of RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 50 μM of 2-mercaptoethanol. On Day 3 of culture, another 10 ml of fresh medium containing rmGM-CSF was added to the dishes. On Days 6 and 8, half of the medium was replaced by fresh complete medium containing rmGM-CSF. On Day 10, the nonadherent cells were collected and used as immature BMDCs. Other collected cells were cultured for 2 days in another 10 ml of fresh medium containing 100 units/ml of rmGM-CSF and 500 units/ml of rmTNF-α. On day 12, the nonadherent cells were collected for both a flow cytometric analysis and genetic modification and then were used as mature BMDCs.

Generation of DCs from iPS cells

The procedure for inducing the differentiation of iPS cells into mature DCs is composed of four steps, as previously described.[13] In brief, in Step 1, iPS cells were suspended in α-MEM supplemented with 20% FBS and seeded onto OP9 cell layers in dishes. On Day 7, the cells were collected for Step 2 of the culture. In Step 2, the harvested cells were suspended in α-MEM supplemented with 20% FBS, 1,000 U/ml of rmGM-CSF and 50 μM of 2-ME and plated onto new OP9 cell layers. On Day 14, the floating cells were collected *via* pipetting for Step 3 of the culture. In Step 3, the cells were transferred to bacteriological Petri dishes (Locus, Tokyo, Japan) without feeder cells and cultured in complete medium

containing 1,000 U/ml of rmGM-CSF. On Day 26, the floating cells were collected for a flow cytometric analysis *via* pipetting for Step 4 of the culture and used as immature iPSDCs. In Step 4, after 2 days, the collected cells were cultured in another 10 ml of fresh medium containing 1,000 units/ml of rmGM-CSF and 5,000 units/ml of rmTNF-α. On Day 28, the nonadherent cells were collected for a flow cytometric analysis and genetic modification and used as mature iPSDCs.

Flow cytometric analysis

The phenotypic analysis of the DCs preparations was performed with a FACSCalibur (Becton Dickinson, San Jose, CA) using the Cell Quest Pro software program, as previously described.[5,6] DCs (1.0×10^6 cells) were incubated with specific antibodies in phosphate buffered saline (PBS) for 30 min at 4°C and rinsed twice. The following monoclonal antibodies (mAb) conjugated with fluorescence isothiocyanate (FITC) were used for staining: anti-mouse CD11c (clone HL3), anti-mouse CD80 (clone 16-10A1), anti-mouse CD86 (clone GL-1), anti-mouse MHC class II ($I\text{-}A^b$) molecules (clone 25-9-17) (all from BD Pharmingen, San Jose, CA), anti-mouse CCR7 (ImmunoDetect, Hardwick, MA) and FITC-conjugated anti-goat IgG polyclonal antibodies (Vector Laboratories, Burlingame, CA). Intracellular staining with anti-melanoma (hgp100) polyclonal antibodies (Acris Antibodies, Herford, Germany) and FITC-conjugated anti-goat IgG polyclonal antibodies (Vector Laboratories) was performed using a Fixation and Permeabilization Solution Kit (BD Biosciences, San Jose, CA).

Adenoviral vector-mediated gene transfer into DCs

Mature DCs were transduced with each recombinant adenoviral vector using a centrifugal method, as previously described.[5,6] Briefly, DCs were mixed with adenoviral vectors at 100 multiplicities of infection (MOI) and then centrifuged at 2,000*g* at 37°C for 2 hr. Our previous studies showed that the optimal MOI for AxCAhgp100 and AxCALacz are 100.[5,6] Therefore, the MOI values of these vectors were fixed at 100 in our study. The DCs were then washed twice with PBS and cultured in complete medium containing 200 units/ml of rmGM-CSF for 48 hr and used as genetically modified DCs, and the hgp100 expression of each DC was quantified using intracellular staining flow cytometry. The abbreviations for the vectors are: BMDCs-hgp100, BMDCs transduced with AxCAhgp100; and iPSDCs-hgp100, iPSDCs transduced with AxCAhgp100.

Assays for cytokine secretion

Genetically modified DCs were seeded at a concentration of 5.0×10^5 cells/well and cultured on a 48-well plate for 48 hr. The supernatants were then harvested, and the murine IL-12 (p70) and interferon (IFN)-γ levels were measured using an mIL-12 (p70) ELISA kit and an mIFN-γ ELISA kit, respectively (Thermo Scientific, Waltham, MA), as previously described.[6]

Trafficking study of subcutaneously injected DCs

We examined whether the iPSDCs were able to migrate to regional lymph nodes as well as BMDCs, following the method described previously.[5] DCs (BMDCs or iPSDCs) were labeled with the GREEN fluorescent 15 dye PKH67 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The cells were washed and incubated with PKH67 staining solution for 5 min. Complete medium containing 10% FBS was added to the cells, and unbound PKH67 was removed using extensive washing with PBS. DCs (1.0×10^6 cells) labeled with PKH67 were injected subcutaneously into the flanks of the mice. After 72 hr, the mice were sacrificed, and the draining inguinal lymph nodes were removed. Some of the harvested lymph nodes were gently homogenized and suspended in PBS. The suspensions were then subjected to a cell imaging analysis to detect fluorescence-positive cells and counterstaining with DAPI (Life Technologies) within the lymph node preparation. This analysis was performed using Cellomics CellInsight (Thermo Scientific) according to the manufacturer's instructions. The other harvested lymph nodes were frozen with O.C.T. Compound (Sakura Finetek Japan, Tokyo) in liquid nitrogen. Some of the frozen tissue sections were fixed with 10% formalin and counterstained with hematoxylin and eosin. The other harvested lymph nodes were gently homogenized and suspended in PBS. The suspensions were subsequently subjected to a flow cytometric analysis to detect fluorescence-positive cells.

MHC Class I tetramer assay and the induction of antigen-specific CTLs and cytotoxicity

To determine whether the administration of DCs (BMDCs or iPSDCs) transfected with AxCAhgp100 would induce hgp100-specific CTLs, DCs were transfected with AxCAhgp100 and AxCALacz (at 100 MOI), as described above. C57BL/6 mice were immunized once *via* subcutaneous injection in the flank with genetically modified DCs (1.0×10^6 cells) suspended in 200 μl of PBS. On Day 14, the spleens were removed, and some of the *in vivo*-primed splenocytes (8.0×10^6 cells/well) were pooled and cocultured in a six-well plate (in complete medium containing 50 units/ml of rmIL-2 at 4 ml/well). After 5 days of coculture, the collected cells were incubated with $H\text{-}2D^b$ hgp100 Tetramer or $H\text{-}2D^b$ Influenza NP Tetramer (MBL, Nagoya, Japan) for 20 min at 4°C in combination with anti-CD8 mAb with FITC (MBL) according to the manufacturer's instructions. The incubated cells were subjected to a flow cytometric analysis. The remaining *in vivo*-primed splenocytes (8.0×10^6 cells/well) were pooled and cocultured with mitomycin C-treated B16 cells (8.0×10^5 cells/well) in a six-well plate (in complete medium containing 50 units/ml of rmIL-2 at 4 ml/well). After 5 days of coculture, the CD8(+) CTLs were sorted from the *in vivo*-restimulated splenocytes using an autoMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and assayed in a 4-hr ^{51}Cr-release assay, as previously described.[5,6]

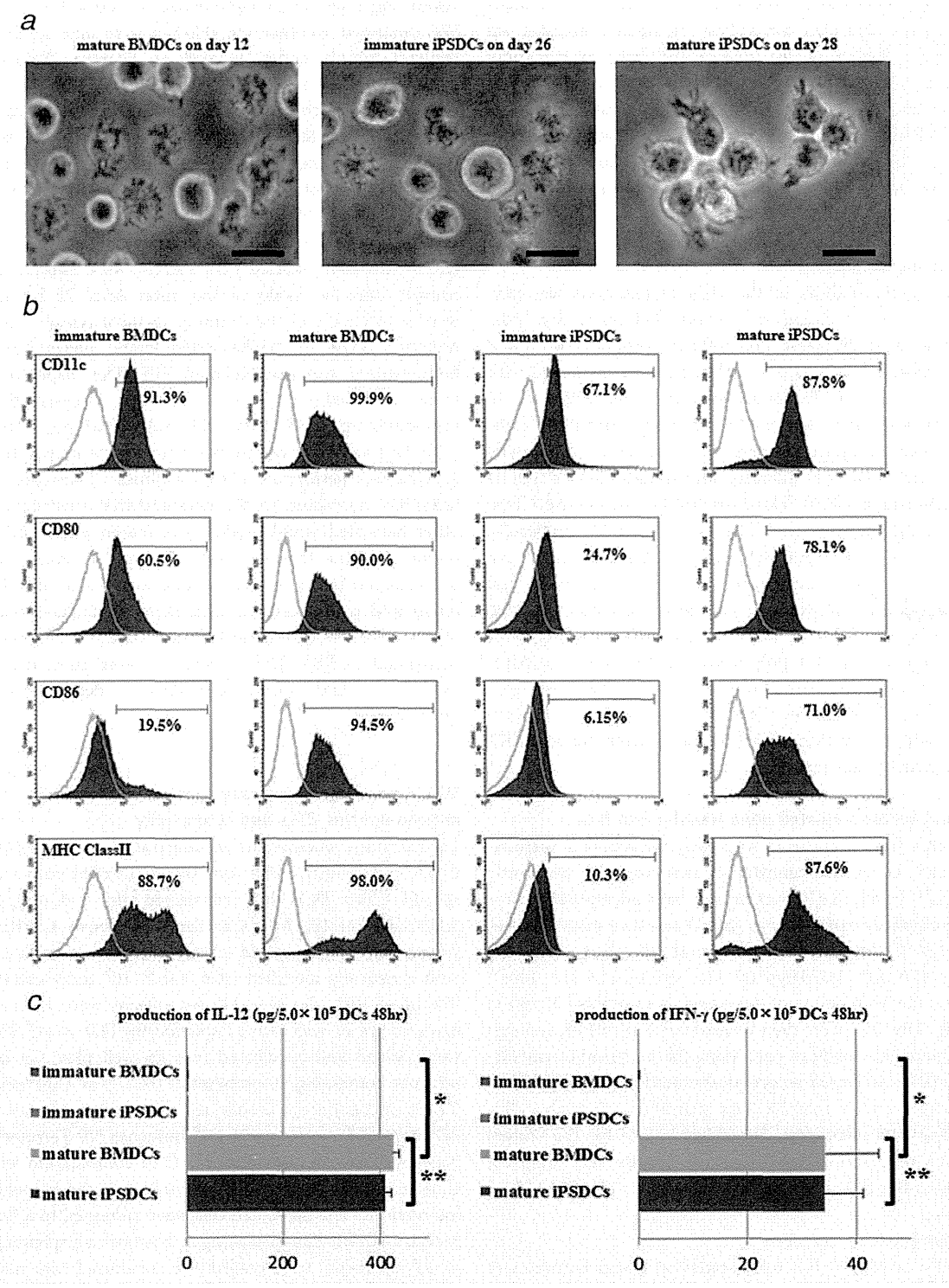

Figure 1. Mature capacity of DCs. (*a*) Morphology of mature BMDCs on Day 12, immature iPSDCs on Day 26 and mature iPSDCs on Day 28. The scale bars represent 20 μm. (*b*) Surface phenotypes of BMDCs and iPSDCs. The staining patterns of specific antibodies (black) and isotype-matched controls (thin lines) are shown in histograms. (*c*) Secretion of IL-12 and IFN-γ from BMDCs and iPSDCs. The results are shown as the mean ± SD ($n = 5$ for each group). *Significantly higher than the immature DCs ($p < 0.0001$). **No significant differences compared to the mature DCs ($p > 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

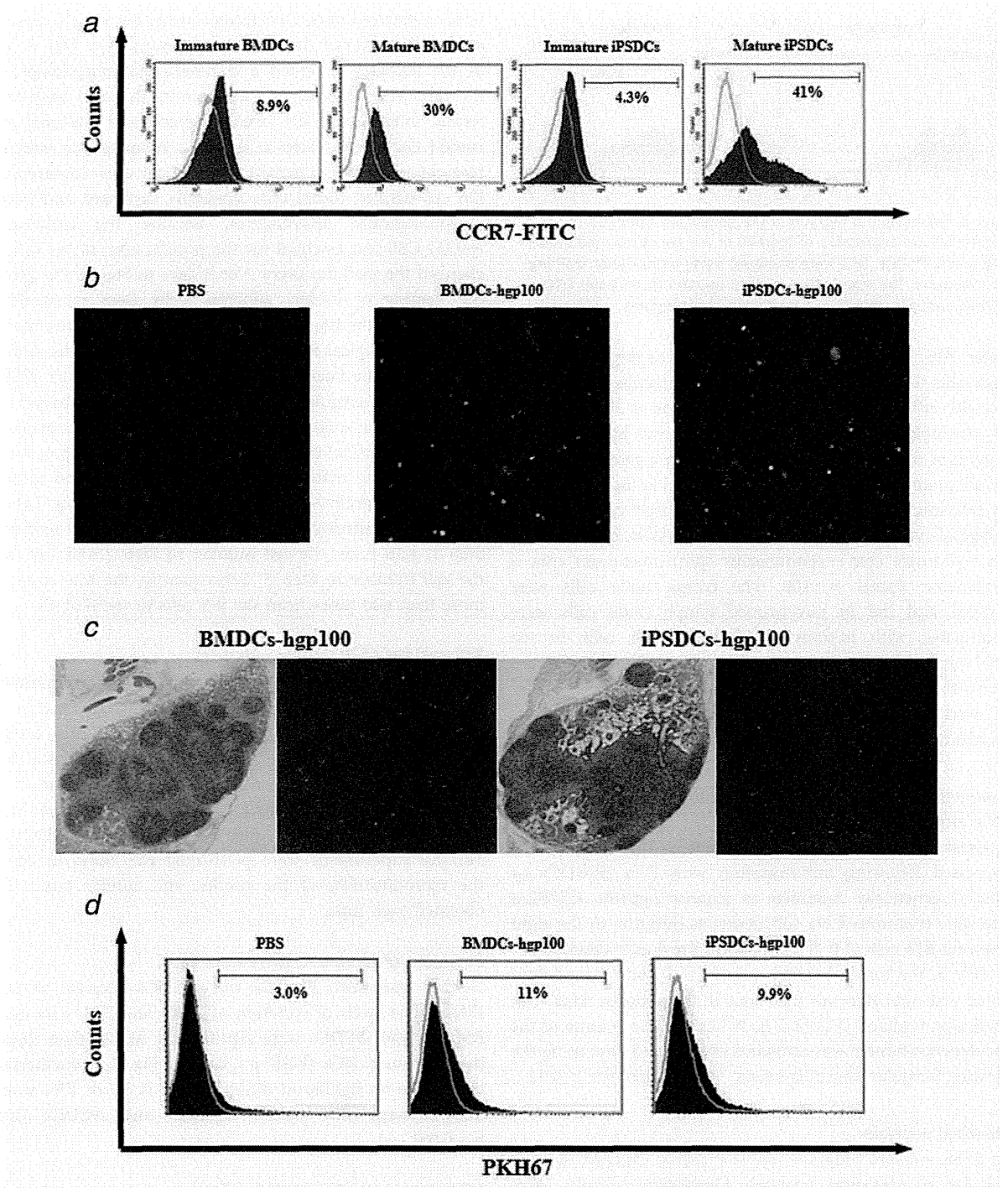

Figure 2. Migratory capacity of DCs *in vivo*. (*a*) BMDCs and iPSDCs were examined for the expression of CCR7 using flow cytometry. The staining patterns of specific antibodies (black) and FITC controls (thin lines) are shown in histograms. (*b*) Single-cell suspensions obtained from the draining lymph nodes were counterstained with the blue fluorescent dye DAPI in the nuclei and analyzed using a cell imaging analysis. Green fluorescent-positive cells and DCs stained with PKH67 were observed in the BMDCs and iPSDCs (image size: 660.48 μm × 660.48 μm). (*c*, *d*) The migratory capacity of DCs *in vivo*. BMDCs-AxCAhgp100 and iPSDCs-AxCAhgp100 were labeled with the fluorescent dye PKH67 and the DCs and PBS were injected s.c. into the lower abdomen of the mice. After 72 hr, (*c*) the draining LNs were observed using fluorescence microscopy of the cryostat sections. (*d*) Single-cell suspensions obtained from the draining LNs were analyzed for labeled cells using flow cytometry. The staining patterns of PKH67 (black) and untreated lymph node-controls (thin lines) are shown in histograms. Histograms represent fluorescence of ungated, total lymph node cell suspensions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]