duodenojejunostomy in relation to DGE. In our RCT comparing the antecolic route with the retrocolic route for duodenojejunostomy after PpPD, DGE occurred in 5% of patients treated using the antecolic route, compared with 50% of those treated using the retrocolic route ($P = 0.0014$) [47]. An interim analysis using Bonferroni's method and involving 20 patients per arm was planned, although the adequate sample size for this RCT was calculated to be a total of 116 patients (58 per arm). This interim analysis clearly indicated a significant benefit of the antecolic route over the retrocolic route with regard to the incidence of DGE, resulting in a decision to terminate the RCT, based on statistical and ethical factors. The antecolic route for duodenojejunostomy during PpPD significantly reduced the incidence of DGE.

The antecolic route for duodenojejunostomy during PpPD may be superior to the retrocolic route with regard to the incidence of DGE for several reasons. For example, the antecolic route may reduce the incidence of DGE by changing the anastomosis position, such as by causing transient torsion or angulation of the anastomosis, by setting the stomach vertically in the left abdomen [11]. Several studies have suggested that gastric dysrhythmia secondary to other abdominal complications, such as a pancreatic fistula or intra-abdominal abscess, increased the incidence of DGE [11, 12, 41–43]. The use of the antecolic route for an anastomosis may avoid clinical inflammation associated with pancreatic fistula or intra-abdominal abscess better than the retrocolic route.

On the other hand, Chijiiwa et al. [48] reported that DGE occurred in 6% of patients operated on using the antecolic route, compared with 22% of those operated on using the vertical retrocolic route ($P = 0.34$); the difference between the antecolic route and the vertical retrocolic route concerning the incidence of DGE was not significant. For the vertical retrocolic route, the left side of the transverse mesocolon (left side of the middle colic vessels) was opened, and the duodenum was brought down together with the gastric antrum in a straight, vertical manner.. As a result of the above findings, Chijiiwa et al. therefore suggested that the two routes after PpPD were similar concerning DGE.

Kim et al. [39] suggested that DGE may be caused by pylorospasms secondary to inadvertent surgical injuries to the branches of the vagus nerve innervating the pyloric region. Two reports describe surgical techniques to manage pylorospasms due to denervation after PpPD, including mechanical dilatation of the pyloric ring and pyloromyotomy [38, 39]. One of these studies suggested that the addition of pyloric dilatation to the PpPD procedure reduced the incidence of DGE from 26 to 6.5% ($P < 0.05$) compared with conventional PpPD [38]. On the other hand, in the other study, the addition of pyloromyotomy during PpPD reduced the incidence of DGE from 21 to 2% ($P < 0.01$) compared with conventional PpPD [39]. However, these two studies were not RCTs. To avoid bias issues, RCTs should be performed to conclusively determine the better surgical procedure.

In two RCTs comparing pancreaticogastrostomy (PG) with pancreaticojejunostomy (PJ), the incidence of DGE was significantly lower after PG during PD than after PJ during PD or PpPD [49, 50]. Bassi et al. [49] reported that DGE, biliary fistula, and postoperative collections were significantly reduced in the PG group, although there were no significant differences concerning the overall complications and incidence of pancreatic fistula between subjects treated with PG and those treated with PJ. Fernández-Cruz et al. [50] reported that the incidence of DGE and pancreatic fistula was significantly reduced in their PG group, compared with the PJ group, due to a new procedure. They proposed that the smaller postsurgical peri-anatomic space in PG compared with PJ may reduce postoperative collections and DGE secondary to inflammation due to fluid collection. On the other hand, according to another two RCTs comparing PG with PJ, there was no significant difference between the incidence of DGE after PG and that after PJ [51, 52].

Postoperative management for preventing DGE

Gastric atony, which plays a role in the pathogenesis of DGE after PpPD, seems to result from a reduction in the circulating levels of motilin, a hormone primarily localized in the enterochromaffin cells of the duodenum and proximal small intestine. Erythromycin and related 14-member macrolide compounds act as motilin agonists by binding to motilin receptors and initiating the phase 3 activity of the interdigestive migratory motor complex (MMC). Two RCTs showed that the administration of erythromycin after PpPD reduced the incidence of DGE [34, 43]. First, Yeo et al. [43] performed a randomized placebo-controlled trial in 118 patients undergoing PD. They reported that high doses (200 mg) of intravenous erythromycin lactobionate every 6 h from postoperative day (POD) 3 to POD 10 after PD reduced the incidence of DGE from 30 to 19%. The administration of a high dose in non-infected patients may induce strong, prolonged bursts of antral contraction, which are not propagated to the small bowel, although no major adverse reactions to erythromycin were observed in this study. Ohwada et al. [34] also reported the incidence of DGE to be lower (14.3%) in the erythromycin group (administration of 1 mg/kg of intravenous erythromycin lactobionate every 8 h from POD 1 to POD 14) compared with 57.1% in the control group ($P = 0.04$).

Conclusion

Several RCTs have clarified two procedures; namely, PD and PpPD, to be equally effective for periampullary tumors

with regard to morbidity, mortality, QOL, and survival. However, the most effective way to prevent DGE remains controversial. The findings of RCTs investigating techniques such as the antecolic route of duodenojejunostomy or PrPD are helping to reduce the incidence of DGE. Further studies should be performed to clarify the long-term QOL and/or nutritional status resulting after the use of these techniques.

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Triple Positive Tumor Markers for Hepatocellular Carcinoma Are Useful Predictors of Poor Survival

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Objective: To determine the importance of the expression pattern of multiple tumor markers for hepatocellular carcinoma (HCC) with regard to the tumor malignancy and patient survival.

Background: Several studies have indicated that HCC tumor markers, including alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP and des-γ-carboxy prothrombin were predictors of HCC malignancy. However, few reports have shown the relevance of the expression pattern of these 3 tumor markers with regard to patient prognosis. We herein reported the influence of the expression pattern of these 3 tumor markers on HCC malignancy and patient prognosis.

Methods: This retrospective study analyzed 185 patients who underwent hepatectomy for HCC between January 1999 and May 2009. The relationships between clinical parameters and these 3 tumor markers were analyzed. Cox proportional hazards regression analyses were performed to estimate risk factors for recurrence and survival. Furthermore, the relationships between pathological parameters and the expression patterns of the 3 tumor markers were analyzed.

Results: From clinical parameters, expression patterns of 3 tumor markers were related to maximum tumor size and macrovascular invasion in image findings. Multivariate analyses revealed independent risk factors for recurrence or survival to be the Child-Pugh score, the presence of multiple tumors, and triple positive tumor marker expression. From pathological findings, microvascular invasion and an Edmondson-Steiner classification of III or IV were related to the expression patterns of the 3 tumor markers.

Conclusions: Triple positive tumor markers for HCC showed poor prognosis and invasive characteristics in pathological findings. Examination of these markers would be useful for predicting the degree of HCC malignancy.

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Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide, and it is especially common in southern and eastern Asia. It is also the third most common cause of death from cancer.[1,2] In general, the most potent diagnostic methods for HCC are imaging studies, such as ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), and angiography. Tumor staging is conducted predominantly based on tumor number, size, macrovascular invasion, and extrahepatic metastasis by these imaging studies according to UICC-TNM classification.[3]

In addition to imaging related diagnoses, the 3 tumor markers are clinically used for HCC diagnosis in Japan; Alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and des-γ-carboxy prothrombin (DCP).

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Some studies have indicated that tumor markers are not only diagnostic tools for HCCs, but also can predict the degree of HCC malignancy.[4–6] However, the cutoff value of each tumor marker for predicting prognosis was still controversial, and there have been few reports which have investigated the importance of the expression patterns of multiple tumor markers for HCC on tumor characteristics and patient prognosis.

We hypothesized that the degree of HCC malignancy would progress according to the number of tumor markers that were elevated (AFP, AFP-L3, and DCP), and our aim was to investigate the influence of the expression pattern of these 3 tumor markers on the prognosis and clinicopathological parameters of HCC.

PATIENTS AND METHODS

Patients and Diagnosis

Between January 1999 and May 2009, 220 patients underwent a hepatectomy at Wakayama Medical University Hospital (WMUH), and were diagnosed with HCC by histological findings. Of these, 17 patients did not undergo a thorough histological examination because of preoperative treatment, 6 patients were diagnosed with combined hepatocellular and cholangiocarcinoma by histological findings, 1 patient was categorized as having a class C Child-Pugh score and 11 patients were lost to follow-up. These 35 patients were excluded from the present study, and the remaining 185 patients were enrolled in this retrospective study. All of the patients underwent several imaging studies, such as ultrasonography, dynamic CT, enhanced MRI and CT during arterial portography for diagnosis and staging of HCC before surgery. For the pathological diagnosis, histological grade of HCC was determined according to the Edmondson-Steiner classification.[7] The severity of liver disease (liver fibrosis staging and hepatitis activity grading) was also evaluated according to the criteria proposed by the Metavir cooperative study group.[8]

This retrospective study was conducted in accordance with the Declaration of Helsinki and the "ethical guidelines for clinical studies" of the ministry of Health, Labor and Welfare in Japan and also the guidelines of the Ethical Committee on Clinical Investigation of Wakayama Medical University Hospital.

Tumor Markers

AFP, AFP-L3, and DCP were measured at the time of preoperative examinations. The serum AFP level was determined by chemiluminescent enzyme immunoassay (Siemens Immulite AFP IV, Mitsubishi Chemical Medience, Tokyo, Japan), the serum AFP-L3 level was expressed as a percentage of the total AFP (AFP-L3 / total AFP $\times$ 100) by lectin affinity electrophoresis coupled with antibody-affinity blotting (AFP-L3 Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and serum DCP was determined by chemiluminescent enzyme immunoassay (Lumipulse PIVKA-II Eisai, Eisai, Tokyo, Japan). The upper normal ranges of AFP, AFP-L3, and DCP in our institution were 20ng/mL, 10% and 40 mAU/mL, respectively. In this study, tumor markers that were higher than the upper normal range were defined as positive markers for HCC. Tumors were classified according to the number of positive tumor markers

as "triple negative," "single positive," "double positive," or "triple positive."

Treatment and Surveillance

At our institution, anatomical resections were basically planned for HCC if the liver function was well preserved and the estimated remnant liver volume met our criteria established previously.[9,10] In brief, our criteria is based on the formulations established by multiple regression analysis as follows: liver failure score = 164.8 − 0.58 × (ALB) − 1.07 × (HPT) + 0.062 × (AST) − 685 × (ICGK) − 3.57 × (OGTT.LI) + 0.074 × (RW) and logit score = 4.15 + 0.03 × (HA) − 0.16 × (remnantVol%), where ALB is serum albumin level (g/dL), HPT is hepaplastin test (%), AST is serum aspartate aminotrasnferase level (IU/L), ICGK is ICG clearance rate, OGTT.LI is blood glucose at 60 and 120 minutes after 75 g glucose oral intake, RW is the estimated resection liver volume, HA is serum hyaluronic acid level, and remnantVol% is the estimated remnant liver volume ratio. The logit score was specially adapted for the patient planned for lobectomy.[10] The value less than 25 of the liver failure score and the value less than 0 of the logit value were estimated as safe liver resection procedure.[9,10]

If any planned liver resection procedure did not meet our criteria, the patient underwent a treatment other than surgery. In this study, anatomical resections (segmental, sectional resection, and lobectomy or more) were performed in 119 patients. Nonanatomical resections were performed in 60 patients.

All of the patients were followed up by administering blood examinations and ultrasonography or abdominal contrast enhanced CT at WMUH every 2 to 3 months after surgery. When a recurrence of HCC was detected, patients received further treatment for HCC, such as repeat hepatectomy, ablation therapy, or transarterial chemoembolization (TACE). After treatment for recurrent lesions, the same surveillance was performed.

Statistical Analysis

Dichotomous variables were compared using the χ^2 test, and continuous variables were compared using *t* tests. Recurrence free survival and disease-specific survival were analyzed using the Kaplan-Meier method and differences were analyzed using the logrank test.

To estimate the risk factors for recurrence and survival by Cox proportional hazards regression analysis, 18 variables were analyzed as follows: age, sex, presence of hepatitis B surface antigen (HBsAg), presence of hepatitis C antibody (HCVAb), Child-Pugh score, serum concentration of albumin, total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), prothrombin time, indocyanine green dye retention rate at 15 minutes ($ICGR_{15}$), platelet count (PLT), number of tumors, maximum tumor size, fibrotic capsule formation and macrovascular invasion from the image findings, liver resection procedure, and triple positive tumor marker expression. In performing Cox proportional hazards regression analysis, continuous variables were converted into binary. The cutoff values were defined as follows; albumin, total bilirubin, and prothrombin time were basically based on the Child-Pugh score.[11] Tumor size was based on the UICC-TNM classification.[3] PLT was based on the value predicting cirrhosis. ICG R_{15}, AST, and ALT were based on the median values in this study. All analyses were performed using the SPSS software program ver.18.0 (SPSS Inc, Chicago, IL). $P < 0.05$ were considered to be significant.

RESULTS

Patient Characteristics and Tumor Markers

Among the 185 patients, there were 146 men and 39 women ranging in age from 33 to 89 years, with a mean ± SD of 67 ± 10 years. The median follow-up was 34 months (range, 2–112 months). During the follow-up period, 47 patients died of recurrence and progression of HCC, 5 patients died of hepatic failure, 3 patients died of gastrointestinal bleeding, 1 patient died of cerebral infarction, 1 patient died of esophageal carcinoma, and 1 patient died of a superior mesenteric artery embolism.

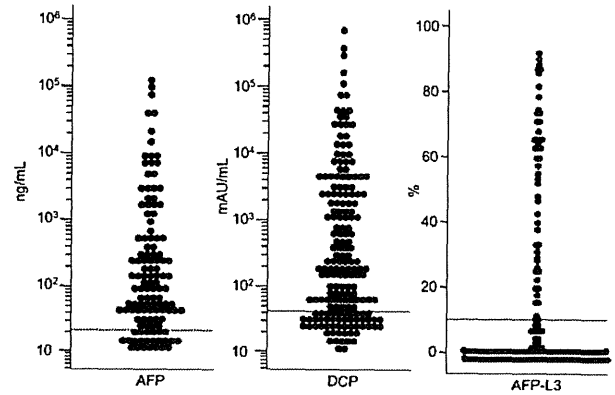

FIGURE 1. Distribution of the 3 tumor markers; alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and des-γ-carboxy prothrombin (DCP). The serum AFP and DCP levels are provided on a log scale. The dotted lines are the upper normal ranges of each tumor marker.

The distribution of serum AFP, AFP-L3, and DCP levels is presented as a dot plot (Fig. 1). The median values of serum AFP, AFP-L3, and DCP levels were 31.0 ng/mL, less than 0.5%, and 195 mAU/mL, respectively. Serum AFP, AFP-L3, and DCP levels over the upper normal ranges were observed in 100 patients (54.1%), 52 patients (28.1%), and 134 patients (72.4%), respectively. Table 1 shows the patients' background and clinical characteristics according to the elevation of each tumor marker. Positive expression of HCV Ab, prothrombin time, AST, and PLT were significantly higher in patients with elevated levels of AFP. The number of tumors, maximum tumor size, presence of macrovascular invasion in image findings, and anatomical liver resection were significantly higher in patients with elevated levels of AFP-L3. The PLT, maximum tumor size, presence of macrovascular invasion in image findings and anatomical liver resection were significantly higher in patients with elevated levels of DCP.

Relationships Between Patient Backgrounds and the Number Of Positive Tumor Markers

According to qualitative evaluation of these 3 tumor markers, 75 patients were single positive [AFP (n = 16); DCP (n = 58); AFP-L3 (n = 1)], 47 patients were double positive (AFP and DCP (n = 35); AFP and AFP-L3 (n = 10); DCP and AFP-L3 (n = 2)], 39 patients were triple positive and 24 patients were triple negative. Among these 4 groups, no significant differences were observed in sex, age, HBsAg, HCVAb, Child-Pugh score, serum concentration of albumin, total bilirubin, AST and ALT, prothrombin time, $ICGR_{15}$, PLT, number of tumors, or fibrotic capsule formation (data not shown). On the contrary, a tumor size larger than 5 cm, the presence of macrovascular invasion, and anatomical liver resection were different among the 4 groups (Fig. 2).

Relationships Between Patient Prognosis and the Number Of Positive Tumor Markers

The recurrence-free survival and disease-specific survival of the patients were also analyzed (Figs. 3 and 4). Recurrence-free

TABLE 1. Patient Backgrounds by Their Expression of the 3 Tumor Markers for HCC

	AFP		AFP-L3		DCP	
Variable	**<20 ng/mL**	**≥20 ng/mL**	**<10%**	**≥10%**	**<40mAU/mL**	**≥40mAU/mL**
Sex						
Male/Female	76/9	70/30	106/27	40/12	42/9	104/30
Age	68 ± 9	66 ± 10	67 ± 9	65 ± 11	67 ± 10	67 ± 10
HbsAg						
Negative/Positive	74/11	78/22	112/21	40/12	41/10	111/23
HCVAb						
Negative/Positive	48/37	35/65*	61/72	22/30	18/33	65/69
Child-Pugh score						
Class A/Class B	77/8	91/9	119/14	49/3	45/6	123/11
Albumin (mg/dL)	3.9 ± 0.5	3.9 ± 0.5	3.9 ± 0.5	3.9 ± 0.5	3.8 ± 0.5	4.0 ± 0.5
Total bilirubin (mg/dL)	0.9 ± 0.4	0.9 ± 0.4	0.9 ± 0.4	0.8 ± 0.3	1.0 ± 0.5	0.9 ± 0.3
Prothrombin time (%)	87.1 ± 12.8	82.9 ± 13.4*	84.9 ± 12.7	84.6 ± 14.6	84.6 ± 11.1	84.9 ± 14.0
$ICGR_{15}$ (%)	13.2 ± 7.4	14.2 ± 7.9	14.3 ± 7.8	12.3 ± 7.1	15.6 ± 8.1	13.0 ± 7.4
AST (IU/L)	47.3 ± 23.5	58.9 ± 34.3*	51.9 ± 30.1	57.8 ± 30.7	57.2 ± 29.4	52.2 ± 30.7
ALT (IU/L)	50.5 ± 32.6	57.2 ± 41.1	55.2 ± 40.4	51.2 ± 28.8	61.1 ± 38.9	51.4 ± 36.7
PLT ($\times 10^4/mm^3$)	18.9 ± 14.7	14.9 ± 6.0*	16.9 ± 12.3	16.4 ± 6.9	13.7 ± 5.4	17.9 ± 12.4*
Number of tumors†						
Single/Multiple	60/25	64/36	95/38	29/23*	36/15	88/46
Tumor size†						
≤5 cm/>5 cm	55/30	67/33	94/39	28/24*	44/7	78/56*
Fibrotic capsule formation†						
Absent/Present	13/72	18/82	21/112	10/42	8/43	23/111
Macro-vascular invasion†						
Absent/Present	72/13	72/28	116/17	28/24*	47/4	97/37*
Liver resection						
Anatomical/Nonanatomical	54/31	65/35	77/56	42/10*	22/29	97/37*

Data are expressed as the means ± SD.
Anatomical resection indicates subsegmentectomy, segmentectomy, and lobectomy or more.
*$P < 0.05$ by χ^2 test or t test.
†Evaluated based on imaging findings.
ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B virus surface antigen; HCVAb, anti-hepatitis C virus antibody; $ICGR_{15}$, indocyanine green dye retention rate at 15 min; PLT, platelet count.

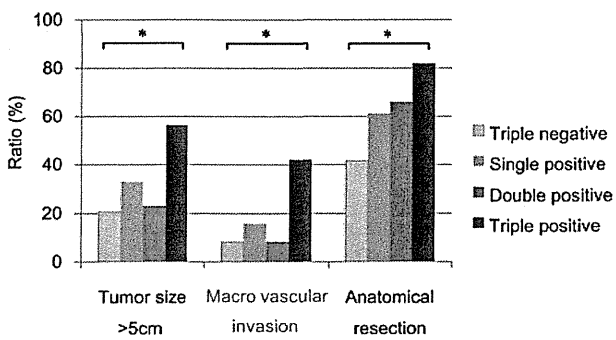

FIGURE 2. The relationships between clinical parameters and the positive patterns of the 3 tumor markers. *$P < 0.05$ by χ^2 test.

survival rates at 2 years after hepatectomy were 19.4% in triple positive, 38.2% in double positive, 55.5% in single positive, and 50.7% in triple negative patients. Disease-specific survival rates at 5 years after hepatectomy were 35.9% in triple positive, 54.7% in double positive, 82.9% in single positive, and 62.8% in triple negative patients. Both recurrence-free and disease-specific survival curves revealed that the patients with triple positive tumor markers had a significantly worst prognosis.

Factors Associated With Recurrence-free Survival Rates

The factors associated with recurrence-free survival were evaluated by univariate and multivariate analyses (Table 2). Univariate analysis revealed that the Child-Pugh score of class B, prothrombin time 80% or less, PLT less than $10 \times 10^4/mm^3$, presence of multiple tumors, presence of macrovascular invasion from image findings and triple positive of tumor markers were significant variables. Multivariate analysis revealed that a Child-Pugh score of class B [relative risk (RR) 2.00, 95% confidence interval (95% CI) 1.03–3.87], the presence of multiple tumors (RR 2.53, 95% CI, 1.73–3.71), and triple positive markers for HCC (RR 1.78, 95% CI, 1.10–2.86) were independent risk factors for recurrence.

Factors Associated With Disease-specific Survival Rates

The factors associated with disease-specific survival were also evaluated by univariate and multivariate analyses (Table 3). Univariate analysis revealed 5 significant variables; Child-Pugh score of class B, the presence of multiple tumors, tumor size larger than 5 cm, the presence of macrovascular invasion, and triple positive of tumor markers for HCC. In a multivariate analysis, 15 outcome events are generally needed to obtain 1 independent variable. As there were 48 survival outcomes in this study, the top 3 variables obtained by univariate analysis; triple positive tumor markers, the presence of macrovascular invasion, and the number of tumors, were entered into multivariate

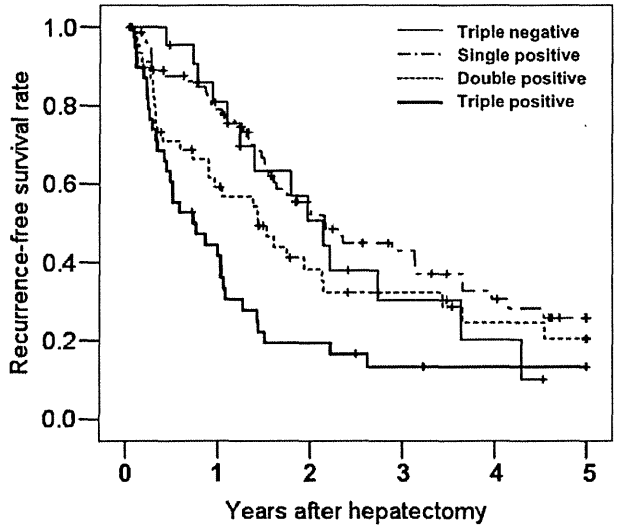

FIGURE 3. Recurrence-free survival curves among patients with different numbers of positive tumor markers. The triple positive group had a significantly shorter recurrence-free interval compared to the triple negative, single positive, and double positive groups (*P* values were 0.02, < 0.001, and 0.045, respectively).

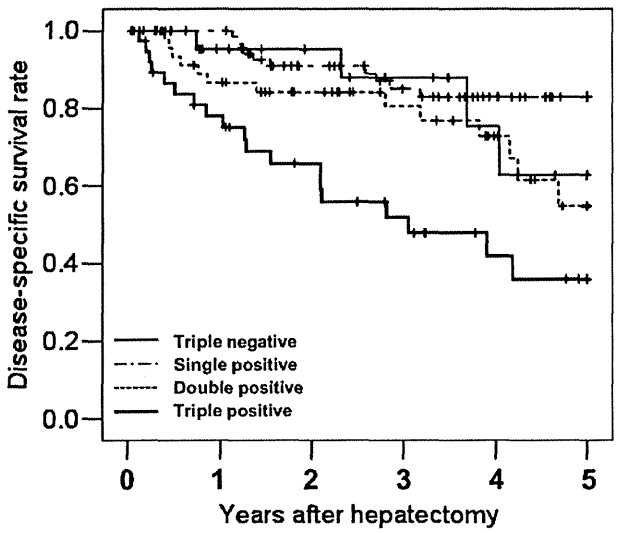

FIGURE 4. Disease-specific survival curves among patients with different numbers of positive tumor markers. The triple positive group had a significantly worse survival compared to the triple negative, single positive, and double positive groups (*P* values were 0.047, <0.001, and 0.02, respectively).

analysis, which revealed that the presence of multiple tumors (RR 2.38, 95% CI, 1.31–4.33) and triple positive markers for HCC (RR 2.41, 95% CI, 1.20–4.83) were independent risk factors for disease-specific survival.

Comparison of Pathological Findings Among Number of Positive Tumor Markers

Pathological findings obtained after hepatectomy were compared among number of positive tumor markers (Fig. 5). The existence of microvascular invasion (triple negative, 16.7% (4/24); single positive, 29.3% (22/75); double positive, 46.8% (22/47); triple positive, 56.4% (22/39)) and an Edmondson-Steiner classification of III or IV [triple negative, 12.5% (3/24); single positive, 13.3% (10/75); double positive, 25.5% (12/47); triple positive, 35.9% (14/39)] were significantly different among these 4 groups.

DISCUSSION

In this study, we showed that the patients who were triple positive for 3 tumor markers of HCC, namely AFP, AFP-L3, and DCP had shorter survival and more pathologically invasive characteristics.

Combinations of these 3 tumor markers are frequently used for detection of tumors in Japan and their usefulness has been reported previously.[12–14] Although previous articles showed the influence of each HCC marker on prognosis, most of these reports have examined the relationship between individual HCC tumor markers and prognosis.[4,15,16] There were few studies that investigated the significance of expression pattern of multiple tumor markers. One study reported that positivity for all 3 HCC tumor markers could predict the HCC patient's outcome.[17] However, their study included various treatment modalities such as hepatectomy, ablation therapy, and TACE. Their result might be biased by the treatment modalities and might be difficult to interpret the results for patients who underwent just a hepatectomy. In this study, we limited the patients to the hepatectomy population and clearly revealed the prognostic significance of triple positive tumor marker expression.

In this study, as shown in Table 1, the patients with AFP-L3 positive had a higher frequency of multiple tumors, large tumor size, and macrovascular invasion. Therefore, the following question would arise; how do the recurrence and survival curves compare between the triple positive patients and those who are just AFP-L3 positive? As previously described, the 5-year disease-specific survival rate and the 2-year recurrence-free survival rate of the triple positive patients were 35.9% and 19.5% in this study. Although those of the 13 patients with AFP-L3 positive but not triple positive tumor markers were 80.2% and 40%, respectively and revealed to be statistically different in 5-year disease-specific survival ($P = 0.03$), although a significant difference in 2-year recurrence-free survival was not seen ($P = 0.09$) (data not shown). Therefore, estimating just AFP-L3 would not be sufficient to evaluate the prognostic impact for HCC.

In this study, we defined cutoff values of the 3 tumor markers as above the upper normal limit. Previous reports have set the cutoff values as follows: (1) the median value, (2) the value calculated from receiver–operator characteristic (ROC) analysis, (3) the value of the upper or lower normal limit, and (4) values based on other reports.

First, in terms of the median value, we were able to calculate the median value of AFP and DCP. However, we could not evaluate the median value of AFP-L3, because most of the patients in this study had less than the minimum detectable limit for AFP-L3. Therefore, the median value could not be used for the cutoff value for the definition of positivity.

Second, ROC analysis in this study showed that the areas under the curve (AUCs) of AFP, AFP-L3, and DCP were 0.59, 0.58 and 0.55, respectively. Because the AUC values need to be at least more than 0.7 for significance discrimination, a cutoff value calculated from ROC analysis could not be used in this study.[18]

Moreover, referring to other reports is a good method for determining a cutoff value. However, different cutoff values were set in various reports, ranging from 20 to 400ng/mL for AFP[19–23], 10% to 15% for AFP-L3[23,24] and 40 to 100 mAU/mL for DCP.[13,25]

In addition, chronic hepatitis can affect serum AFP levels. However, a value of 16 ng/mL for serum AFP could distinguish HCC from chronic hepatitis.[26] Therefore, setting the cutoff value

TABLE 2. Univariate and Multivariate Analyses for Recurrence-free Survival in 185 Patients Undergoing Hepatectomy for Hepatocellular Carcinoma

		Univariate analysis		Multivariate analysis	
Variable	**n**	**RR (95% CI)**	***P***	**RR (95% CI)**	***P***
Sex					
Female	39	1			
Male	146	0.83 (0.55–1.27)	0.39		
Age					
<68 years	90	1			
≥68 years	95	0.82 (0.57–1.17)	0.27		
HBsAg					
Negative	152	1			
Positive	33	1.21 (0.77–1.88)	0.41		
HCVAb					
Negative	83	1			
Positive	102	1.15 (0.80–1.65)	0.45		
Child-Pugh score					
Class A	168	1			
Class B	17	1.87 (1.05–3.34)	0.03	2.00 (1.03–3.87)	0.03
Albumin					
>3.5 g/dL	150	1			
≤3.5 g/dL	35	1.39 (0.90–2.15)	0.13		
Total bilirubin					
<1.0 mg/dL	113	1			
≥1.0 mg/dL	72	1.17 (0.82–1.68)	0.38		
Prothrombin time					
>80%	121	1			
≤80%	64	1.52 (1.06–2.18)	0.02	1.21 (0.81–1.83)	0.34
$ICGR_{15}$					
<12.2%	92	1			
≥12.2%	93	1.33 (0.93–1.91)	0.12		
AST					
<48 IU/L	89	1			
≥48 IU/L	96	1.34 (0.93–1.92)	0.12		
ALT					
<45 IU/L	92	1			
≥45 IU/L	93	0.99 (0.70–1.42)	0.97		
PLT					
$\geq 10 \times 10^4/mm^3$	159	1			
$< 10 \times 10^4/mm^3$	26	1.89 (1.12–3.00)	0.007	1.56 (0.95–2.54)	0.08
Number of tumors*					
Single	124	1			
Multiple	61	2.46 (1.69–3.56)	<0.0001	2.53 (1.73–3.71)	<0.0001
Tumor size*					
≤5 cm	122	1			
>5 cm	63	1.31 (0.90–1.92)	0.16		
Fibrotic capsule formation*					
Present	154	1			
Absent	31	1.45 (0.92–2.28)	0.11		
Macrovascular invasion*					
Absent	144	1			
Present	41	1.65 (1.06–2.54)	0.03	1.16 (0.71–1.90)	0.56
Liver surgery					
Nonanatomical	66	1			
Anatomical	119	1.15 (0.80–1.66)	0.46		
Tumor markers					
Nontriple positive	146	1			
Triple positive	39	1.93 (1.27–2.93)	0.002	1.78 (1.10–2.86)	0.02

Triple positive patients are defined as those whose serum levels of all 3 tumor markers are above the normal range.

*Evaluated based on imaging findings.

ALT indicates serum alanine aminotransferase; AST, serum aspartate aminotransferase; CI, confidence interval; HBsAg, hepatitis B virus surface antigen; HCVAb, anti-hepatitis C virus antibody; $ICGR_{15}$, indocyanine green dye retention rate at 15 min; PLT, platelet count; RR: relative risk.

TABLE 3. Univariate and Multivariate Analyses for Disease-specific Survival in 185 Patients Undergoing Hepatectomy for Hepatocellular Carcinoma

		Univariate analysis		Multivariate analysis	
Variable	**n**	**RR (95% CI)**	***P***	**RR (95% CI)**	***P***
Sex					
Female	39	1			
Male	146	0.63 (0.34–1.19)	0.16		
Age					
<68 years	90	1			
≥68 years	95	0.84 (0.46–1.51)	0.55		
HBsAg					
Negative	152	1			
Positive	33	1.67 (0.86–3.23)	0.13		
HCVAb					
Negative	83	1			
Positive	102	1.25 (0.68–2.29)	0.47		
Child-Pugh score					
Class A	168	1			
Class B	17	2.52 (1.17–5.42)	0.02		
Albumin					
>3.5 g/dL	150	1			
≤3.5 g/dL	35	1.92 (0.99–3.72)	0.054		
Total bilirubin					
<1.0 mg/dL	113	1			
≥1.0 mg/dL	72	0.78 (0.43–1.44)	0.43		
Prothrombin time					
>80%	121	1			
≤80%	64	1.78 (0.99–3.21)	0.056		
$ICGR_{15}$					
<12.2%	92	1			
≥12.2%	93	0.84 (0.46–1.53)	0.57		
AST					
<48 IU/L	89	1			
≥48 IU/L	96	1.35 (0.74–2.47)	0.33		
ALT					
<45 IU/L	92	1			
≥45 IU/L	93	1.05 (0.58–1.89)	0.87		
PLT					
$\geq 10 \times 10^4/mm^3$	159	1			
$< 10 \times 10^4/mm^3$	26	1.83 (0.91–3.70)	0.09		
Number of tumors*					
Single	124	1			
Multiple	61	2.58 (1.43–4.66)	0.002	2.38 (1.31–4.33)	0.005
Tumor size*					
≤5 cm	122	1			
>5 cm	63	2.30 (1.28–4.15)	0.006		
Fibrotic capsule formation*					
Present	154	1			
Absent	31	1.61 (0.80–3.26)	0.18		
Macro-vascular invasion*					
Absent	144	1			
Present	41	2.92 (1.55–5.50)	0.0009	1.67 (0.80–3.48)	0.17
Liver surgery					
Nonanatomical	66	1			
Anatomical	119	1.57 (0.82–2.99)	0.17		
Tumor markers					
Nontriple positive	146	1			
Triple positive	39	3.10 (1.69–5.67)	0.0002	2.41 (1.20–4.83)	0.01

Triple positive patients are defined as those whose serum levels of all 3 tumor markers are above the normal range.

*Evaluated based on imaging findings.

ALT indicates serum alanine aminotransferase; AST, serum aspartate aminotransferase; CI, confidence interval; HBsAg, hepatitis B virus surface antigen; HCVAb, anti-hepatitis C virus antibody; $ICGR_{15}$, indocyanine green dye retention rate at 15 min; PLT, platelet count; RR, relative risk.

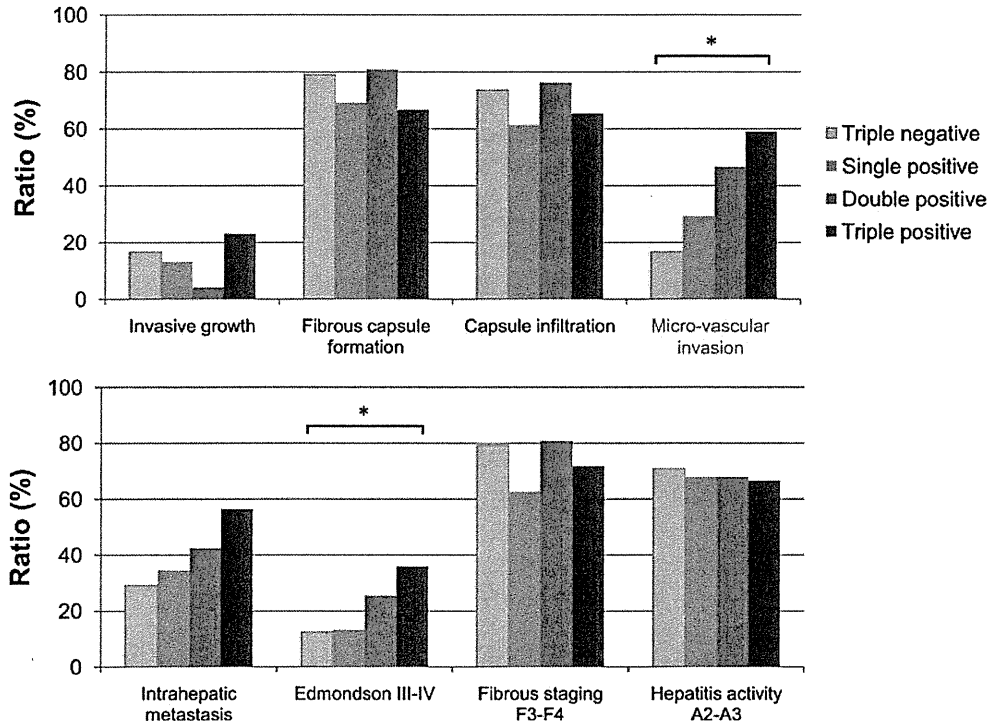

FIGURE 5. The relationships between pathological parameters and the positive expression pattern of the 3 tumor markers. $*P < 0.05$ by χ^2 test.

as the upper normal limit (20 ng/mL for AFP) was appropriate for predicting the prognosis of HCC.

For the reasons stated earlier, the cutoff values of these 3 tumor markers were defined as upper normal limits in this study.

The type of surgical procedure selected was also considered to affect the prognosis, and anatomical resection was reported to have a beneficial effect on prognosis.[27] Although in this study anatomical resection was more common in the "triple positive" group, the prognosis was significantly worse. This might indicate that "triple positive" HCC has a higher degree of tumor malignancy.

This study also revealed pathological differences among the number of positive tumor markers. Indeed, several studies revealed that high serum levels of tumor markers were predictive of portal vein invasion.[4,28] In addition to the previous study, our pathological findings revealed that there is increasing invasiveness and poorer differentiation characteristics in HCC according to the number of positive tumor markers.

In general, vascular invasion has a prognostic impact. However, our result in this study failed to reveal an independent risk factor. In this study, the factor of macrovascular invasion was evaluated by imaging studies. Therefore, microvascular invasions that could not be detected by imaging studies were entered into absent macrovascular invasions in Tables 2 and 3. Therefore, the statistical power of discrimination might be diminished. Furthermore, the triple positive tumor marker, which revealed to represent tumor invasiveness including microvascular invasion, was a stronger factor than the factor of macrovascular invasion evaluated by imaging studies. In this point, the positivity of 3 tumor markers would reflect and involve the prognostic impact of vascular invasion.

In the clinic, our results would be informative for selecting the most appropriate therapeutic modality. If their liver function was well preserved, the patients with triple positive tumor marker might avoid locogerional ablation therapy because of its pathological invasiveness. However, anatomical resection was performed in a large number of triple positive patients in this study and the recurrence rate was also high. Therefore, these patients might be candidates for adjuvant chemotherapies. Otherwise, these patients might be recommended for liver transplantation.

Recently, some other newly biological markers such as Glypican-3 has been identified for HCC.[29] Although these biomarkers are not currently in clinical use, these biomarkers identified by molecular biological methods would reveal biological function of HCC in further analyses and might change the importance of triple positive of AFP, AFP-L3, and DCP as a prognostic marker for patients with HCC after hepatectomy.

In conclusion, a triple positive status for the 3 tumor markers of HCC was an independent risk factor for recurrence and survival, and indicated an increasing presence of vascular invasion and poor differentiation in pathological findings.

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Dendritic cells adenovirally-transduced with full-length mesothelin cDNA elicit mesothelin-specific cytotoxicity against pancreatic cancer cell lines *in vitro*

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ABSTRACT

Mesothelin (MSLN) is an attractive candidate as a molecular target for pancreatic cancer immunotherapy. The purpose of this study was to demonstrate that cytotoxic T lymphocytes (CTLs) generated from peripheral blood mononuclear cells (PBMCs) by stimulation with genetically-modified dendritic cells (DCs) expressing MSLN could produce specific anti-tumor immunity against pancreatic cancer cells endogenously expressing MSLN. MSLN-specific CTLs were generated from PBMCs of healthy donors by *in vitro* stimulation with DCs adenovirally-transduced with the full-length MSLN gene (DC-AxCAMSLN). The cytotoxic activity was tested using a 4-h ^{51}Cr-release assay. The pancreatic cancer cell lines (PK1, CfPAC1, AsPC1), a lymphoblastoid cell lines (LCL) transduced with the MSLN gene, and LCL pulsed with MSLN-epitope peptides were used as target cells. MSLN-specific CTLs induced by *in vitro* stimulation with DC-AxCAMSLN killed pancreatic cancer cell lines expressing MSLN in an HLA-restricted fashion. These CTLs also showed cytotoxic activity against autologous LCL pulsed with multiple MSLN-derived epitope peptides. In addition, $CD8^+$ T cells, as well as $CD4^+$ T cells, sorted from these CTLs showed significant production of interferon-γ when stimulated with DC-AxCAMSLN. The *in vitro* stimulation of PBMCs with DCs transduced with the full-length MSLN gene elicited a potent MSLN-specific cytotoxic activity against pancreatic cancer cell lines endogenously expressing MSLN by recognizing multiple MSLN epitopes and activating both $CD8^+$ T cells and $CD4^+$ helper T cells. These results therefore suggest the potential of developing future clinical applications of the vaccines using genetically-modified DCs expressing MSLN.

1. Introduction

Pancreatic cancer has an extremely poor prognosis, with an overall 5-year survival of 5% [1]. Curative surgery for patients with pancreatic cancer significantly improves their prognosis; however the majority of patients with pancreatic cancer are diagnosed at an advanced stage that makes curative resection very difficult [2]. Chemotherapy using gemcitabine is the standard treatment for unresectable pancreatic cancer at present, although its effects are relatively limited [3]. The development of more effective treatment strategies is therefore urgently needed.

Immunotherapy is a novel approach to the management of pancreatic cancer [4]. The clinical potential of various types of vaccines, such as peptide-based vaccines, dendritic cell vaccines, whole tumor cell vaccines, and recombinant viral- or bacterial-vector based vaccines has been demonstrated in early phase clinical trials [5–10]. The immunological and clinical responses in these studies have been promising, however, they are still insufficient for generating significant clinical benefits. Mesothelin

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(MSLN), a glycosylphosphatidylinositol-linked cell surface glycoprotein, is overexpressed in pancreatic ductal adenocarcinomas, however, is not expressed in normal tissues except mesothelial cells, which makes it an attractive candidate as a molecular target for pancreatic cancer immunotherapy [11–14]. In fact, several early phase clinical trials targeting MSLN have recently been reported, including a peptide vaccine, a DNA vaccine, a recombinant immunotoxin, and a chimeric anti-MSLN monoclonal antibody, and immunological responses and some minor clinical responses have been reported [15–20].

Dendritic cells (DCs) are potent antigen-presenting cells that play a critical role in the initiation of anti-tumor immune responses [21]. We have previously shown that DCs genetically transduced with the full-length tumor-associated antigen (TAA) are promising for cancer vaccine development [22,23]. This genetically-modified DC vaccine therapy has several advantages, including the fact that delivery of a broad repertoire of both major histocompatibility complex (MHC) class I and class II restricted epitopes offers the possibility for polyvalent immunization and synergistic $CD4^+$ and $CD8^+$ T-cell responses. Our previous studies have demonstrated that DCs adenovirally-transduced with natural TAA such as gp70 and carcinoembryonic antigen (CEA) were effective for inducing TAA-specific cytotoxic T lymphocytes (CTLs) and that they elicited potent anti-tumor responses in mouse models [22,23].

The purpose of this study was to determine the usefulness of DCs adenovirally-transduced with the whole human MSLN gene as a novel vaccine for patients with pancreatic cancer. We investigated whether these genetically-modified DCs expressing MSLN can induce cytotoxic T lymphocytes (CTLs) that show MSLN-specific cytotoxic activity against pancreatic cancer cells endogenously expressing MSLN, while also trying to clarify whether they can simultaneously induce MSLN-specific $CD4^+$ helper T cells *in vitro*.

2. Materials and methods

2.1. Cell lines

The human pancreatic cancer cell lines PK1 (HLA-A24/24), CfPAC1 (HLA-A2/3), and AsPC1 (HLA-A1/26) were purchased from the American Type Culture Collection (Manassas, VA, USA). Autologous Epstein-Barr virus (EBV)-transfected B-lymphoblastoid cells (LCL) were generated from healthy donor peripheral blood mononuclear cells (PBMCs) transformed by EBV, as described previously [24]. The cells were cultured in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Co., Carlsbad, CA), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

2.2. Immunohistochemistry for MSLN

MSLN protein expression was examined by immunohistochemical staining to evaluate the expression pattern of MSLN in 34 consecutive specimens of pancreatic tumors (invasive ductal adenocarcinoma: 10, intraductal papillary mucinous neoplasms (IPMNs) adenoma: 7, carcinoma *in situ*: 7, invasive adenocarcinoma: 10) that were resected at Wakayama Medical University Hospital. Invasive ductal adenocarcinoma is the most common neoplasm of the pancreas, consisting more than 85% of pancreatic tumors. IPMNs are defined as grossly visible, mucin-producing, predominantly papillary epithelial neoplasms arising from the main pancreatic duct or branch ducts. The intraductal components of IPMNs display broad spectrum of dysplasia ranging from adenoma to carcinoma in situ, and one third of IPMNs have an associated invasive adenocarcinoma and some patients with a non-invasive IPMN subsequently develop invasive ductal adenocarcinoma. Formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mM of sodium citrate buffer (pH 6.0) heated at 121 °C in a steamer for 7 min. The endogenous peroxidase activity was suppressed by a solution of 3% hydrogen peroxide in methanol for 5 min. After being rinsed in Tris-buffered saline (TBS), the sections were incubated with a blocking reagent: Protein block (Dako, Kyoto, Japan) for 20 min at room temperature. The sections were incubated overnight at 4 °C with the primary antibody, a 1:20 dilution of a mouse monoclonal antibody to MSLN (Clone 5B2; LAB VISION, Fremont CA, USA). After rinsing in TBS, the primary antibody was visualized using the Histofine Simple Stain PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's instruction manual. The sections were developed in DAB at room temperature, and counterstained with Mayer's hematoxylin. The immunolabeling of >10% of the neoplastic cells was defined as positive.

2.3. Generation of human DCs

Monocyte-derived DCs were used as antigen-presenting cells to induce CTL responses against MSLN. DCs were generated *in vitro* from the peripheral blood of healthy volunteers. PBMCs isolated from a healthy volunteer's buffy coats using Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, NJ, USA) were separated by adherence to a PRIMARIA™ tissue culture dish (Becton Dickinson) in order to enrich the monocyte fraction. The monocyte-enriched population was cultured for 5 days in AIM-V medium (Invitrogen) containing 2% heat-inactivated autologous serum (AS) supplemented with 1000 U/mL recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc., Minneapolis, MN, USA) and 500 U/mL recombinant human interleukin (rhIL)-4 (kindly provided by Ono Pharmaceutical co., Tokyo, Japan), and then was cultured for additional 24–48 h in the presence of 1000 U/mL rhIL-6 (R&D Systems), 10 ng/mL recombinant human tumor necrosis factor-α (rhTNF-α; R&D Systems), 10 ng/mL rhIL-1β (R&D Systems), and 1 μg/mL prostaglandin E_2 (Sigma–Aldrich) to induce final maturation [25]. The mature DCs were harvested, and the expression of cell surface molecules was analyzed by flow cytometry. Approximately 95% of the cells showed the expression of CD11c, CD80, CD83, and CD86 (data not shown).

2.4. Recombinant adenoviral vector construction

MSLN cDNA, cloned into the pBluescript SK(−) plasmid (provided by Chugai Pharmaceutical co., Ltd., Tokyo, Japan)

was excised by *EcoRI* and *NspI* and blunt-ended, then ligated into the *SwaI* site of cosmid vector pAxCAwt (Takara, Shiga, Japan) to yield pAxCAMSLN. The recombinant adenoviral vector AxCAMSLN, encoding MSLN, was generated by the cosmid-terminal protein complex (COS-TPC) method, as described previously [26]. AxCALacZ encoding β-gal was also generated by the COS-TPC method.

2.5. Gene transduction of DCs

DCs were transfected with AxCAMSLN or AxCALacZ using the centrifugal method [22]. Our previous study showed that the optimal multiplicities of infection (MOIs) was 100 in terms of both the efficiency of transduction and the viability of DCs [27]. Therefore, the MOI for AxCAMSLN was fixed at 100 in this study, and the expression of MSLN on DC-AxCAMSLN was observed to be 61% by flow cytometry, and the viability of DC-AxCAMSLN cells was >90% (data not shown).

2.6. Synthetic peptides

MSLN peptides that bind to HLA-A24 or HLA-A2 molecules at high levels, as described previously [28], were synthesized according to standard solid phase methods, and were purified to >95% purity by high-performance liquid chromatography (Takara). HLA-A24-binding MSLN peptides, FYPGYLCSL ($A24_{(435-443)}$), LYPKARLAF ($A24_{(475-483)}$), and HLA-A2-binding MSLN peptides SLLFLLFSL ($A2_{(20-28)}$), VLPLTVAEV ($A2_{(530-538)}$) were synthesized for the experiments.

2.7. Induction of CTLs from PBMCs

MSLN cDNA-transduced DCs (DC-AxCAMSLN) were irradiated (25 Gy × 3) and transferred into 24-well tissue culture plates (2×10^5 cells/well) and incubated with autologous fresh PBMCs (4×10^6 cells/well) from healthy donors in 1 mL of AIM-V with 2% AS containing rhIL-7 (10 ng/mL; PEPROTECH, Rocky Hill, NJ, USA). RhIL-2 (20 U/mL; PEPROTECH) was added on day 2 in a total volume of 2 mL. On days 7 and 14, the cultures were re-stimulated with DC-AxCAMSLN at a ratio 20:1. Complete medium containing 20 U/mL of rhIL-2 was added every 2–3 days. The cytotoxic activity was analyzed on day 21, after 3 cycles of stimulation.

2.8. Cytotoxicity assay

The cytotoxic activity was tested using a 4-h ^{51}Cr-release assay, as described previously [22]. The percentage of cytotoxic activity was calculated as follows: percentage of lysis = [(cpm of the sample release − cpm of the spontaneous release/(cpm of the maximum release − cpm of the spontaneous release)] × 100. MSLN cDNA-transduced autologous LCL (LCL-AxCAMSLN), LacZ cDNA-transduced LCL (LCL-AxCALacZ), pancreatic cancer cell lines (PK1, CfPAC1, AsPC1), and LCL pulsed with MSLN-epitope peptides (FYPGYLCSL ($A24_{(435-443)}$), LYPKARLAF ($A24_{(475-483)}$), SLLFLLFSL ($A2_{(20-28)}$), and VLPLTVAEV ($A2_{(530-538)}$)) were used as target cells. LCL were pulsed with 20 μg/mL of each MSLN peptides for 16 h at 37 °C.

2.9. Cold target inhibition assay

The antigen specificity of the CTLs induced by the stimulation with genetically-modified DCs expressing MSLN was examined by the cold target inhibition assay. ^{51}Cr-labeled PK1 cells were used as hot targets, and unlabeled LCL-AxCAMSLN or LCL-AxCALacZ were used as cold targets. In a ^{51}Cr-release assay, the effector (CTLs)/hot target (PK1) ratio was fixed at 25, while various cold/hot target ratios were examined.

2.10. Interferon-γ (IFN-γ) release assay

We examined the MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses using an IFN-γ release assay following methods described previously [29]. In brief, $CD4^+$ and $CD8^+$ T cells were isolated from CTLs cultured after 3 cycles of re-stimulation *in vitro* using an autoMACS™ instrument (Miltenyi Biotec, Bergisch Gladbach, Germany). CTLs were washed twice in phosphate-buffered saline containing 0.5% bovine serum albumin (Invitrogen Co., Carlsbad, CA) and 2 mM EDTA. CTLs were then incubated with CD4 or CD8 microbeads (Miltenyi Biotec) for 15 min at 4 °C and then washed prior to separation. Separation was performed using an autoMACS column (Miltenyi Biotec). The column was placed in the magnetic field and magnetically labeled cells were retained in the column and flushed out as positively selected cells when the magnetic field was off. The purity of sorted populations was determined by flow cytometry and was always more than 95% (data not shown). The positively-selected $CD4^+$ and $CD8^+$ T cells (5×10^4) were stimulated with DCs (DC-AxCAMSLN, DC-AxCALacZ, 5×10^3), in a total volume of 200 μL of complete medium in a 96-well round-bottomed plates for 24 h. Thereafter, the supernatants were collected, and the IFN-γ levels were measured using a human IFN-γ Enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Inc., Woburn, MA, USA). Each assay was performed on duplicate samples.

2.11. Statistical analysis

StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA) was used for all statistical analyses. Statistical analysis was performed by a Student's *t*-test. A *p*-value of <0.05 was considered to be significant.

2.12. The experimental procedures

This experiment was approved by the Committee for Recombinant DNA Experiments of Wakayama Medical University. All experiments were performed in accordance within the Guidelines of this Committee. We obtained written informed consent from all healthy donors before experiments.

3. Results

3.1. Immunohistochemistry of pancreatic tumor tissues

Immunohistochemical analysis was performed to investigate the expression pattern of MSLN in pancreatic tumor tissues (including 10 invasive ductal adenocarcinomas, seven adenomas of IPMNs, seven carcinomas in situ of IPMNs, and 10 invasive carcinomas derived from IPMNs) (Table 1). Positive immunostaining was observed in all 10 cases (100%) of invasive ductal adenocarcinoma, and in seven cases (70%) of invasive carcinomas derived from IPMNs. On the other hand, negative immunostaining was observed in adenomas and carcinomas in situ of IPMNs. Even within the same specimen, the expression of MSLN was observed in the invasive component of IPMNs, but was not detected in the non-invasive component (Fig. 1).

3.2. MSLN expression on target cells

We evaluated the expression of MSLN in LCL-AxCAMSLN, LCL-AxCALacZ and pancreatic cancer cell lines (PK1, CfPAC1, AsPC1) by RT-PCR. We observed strong expression of MSLN in PK1, CfPAC1, AsPC1 and LCL-AxCAMSLN, but not in LCL-AxCALacZ (data not shown).

3.3. MSLN-specific CTL responses induced by MSLN cDNA-transduced DCs

CTLs induced by DC-AxCAMSLN from HLA-A24-positive Donors showed cytotoxic activity against autologous LCL-AxCAMSLN generated from each of the donors, whereas they did not show cytotoxic activity against autologous LCL-AxCALacZ (Fig. 2).

Table 1
MSLN immunohistochemistry summary.

	Invasive ductal adenocarcinoma ($n = 10$)	IPMNs		
		Adenoma ($n = 7$)	Carcinoma in situ ($n = 7$)	Invasive carcinoma ($n = 10$)
Negative	0	7 (100%)	7 (100%)	3 (30%)
Positive	10 (100%)	0	0	7 (70%)

MSLN protein expression was examined by immunohistochemical staining to evaluate the expression pattern of MSLN in 34 consecutive specimens of pancreatic tumors. The immunolabeling of >10% of the neoplastic cells was defined as positive.

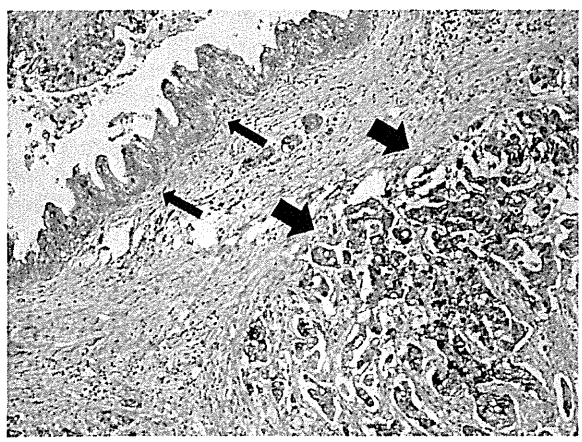

Fig. 1. Immunohistochemical staining for mesothelin protein in the tumor tissue of IPMNs. There is strong labeling of the neoplastic epithelium in the invasive component of IPMNs (thick arrows) but not in the non-invasive component (thin arrows). (magnification 100×).

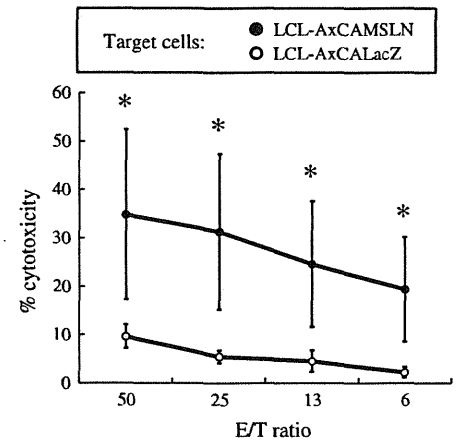

Fig. 2. Cytotoxic activity of CTLs generated from MSLN cDNA-transduced DCs. CTLs cultured after 3 cycles of re-stimulation *in vitro* were used as effectors to test the lysis of autologous LCL-AxCAMSLN and LCL-AxCALacZ. Results were shown as the mean ± SD of seven different donors. *Significant increase of the lysis was shown ($p < 0.01$).

3.4. Cytotoxic activity induced by DC-AxCAMSLN against pancreatic cancer cell lines endogenously expressing MSLN

The CTLs generated by DC-AxCAMSLN from HLA-A24 Donors showed cytotoxic activity against PK1, which endogenously expresses MSLN and possesses the HLA-A24; however they did not show the cytotoxic activity against AsPC1, which endogenously expresses MSLN but does not possess the HLA-A24 (Fig. 3A). The cytotoxic activity against PK1 was suppressed by anti-HLA class I antibody (data not shown). On the other hand, the CTLs generated by DC-AxCAMSLN from HLA-A2 Donors showed cytotoxic activity against CfPAC1 cells, which express MSLN and possess HLA-A2; however, they did not show the cytotoxic activity against AsPC1 cells which endogenously express MSLN but does not possess HLA-A2 nor any shared type of HLA in common with the donors (Fig. 3B). These results suggest that the CTLs induced by genetically-modified DCs expressing MSLN showed MSLN-specific cytotoxic activity that was obviously restricted to the HLA-A types of donors.

3.5. Cold target inhibition assay

To investigate whether the CTL responses against PK1 were dependent on the specificity to MSLN, we carried out a cold target inhibition assay. PK1 cells labeled with $Na_2{}^{51}CrO_4$ were prepared as the hot target, and autologous LCL-AxCAMSLN and LCL-AxCALacZ without labeling were used as the cold targets (inhibitor). The cytotoxic activity of CTLs induced by DC-AxCAMSLN from HLA-A24 positive Donors (Donors 1, 2, and 3) against PK1 was specifically inhibited with the addition of autologous LCL-AxCAMSLN. On the other hand, it was not inhibited by the addition of autologous LCL-AxCALacZ (Fig. 3C).

3.6. MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses induced by DC-AxCAMSLN

To investigate whether MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses in PBMC-derived CTLs were induced by the stimulation with DC-AxCAMSLN, $CD4^+$ T cells and $CD8^+$ T cells were sorted from CTLs, and their ability to produce IFN-γ when they were incubated with DC-AxCAMSLN or DC-AxCALacZ cells was tested. IFN-γ production by the $CD8^+$ T cells incubated with DC-AxCAMSLN was higher than that of $CD8^+$ T cells incubated with DC-AxCALacZ cells. Moreover, IFN-γ production by $CD4^+$ T cells incubated with DC-AxCAMSLN cells was extremely higher than $CD4^+$ T cells incubated with DC-AxCALacZ (Fig. 4).

3.7. The cytotoxic activity induced by DC-AxCAMSLN against autologous LCL pulsed with MSLN-derived epitope peptides

To investigate whether MSLN-derived epitope peptide-specific CTL responses were elicited by the stimulation with DC-AxCAMSLN from PBMCs, the cytotoxic activity of CTLs against epitope peptide-pulsed

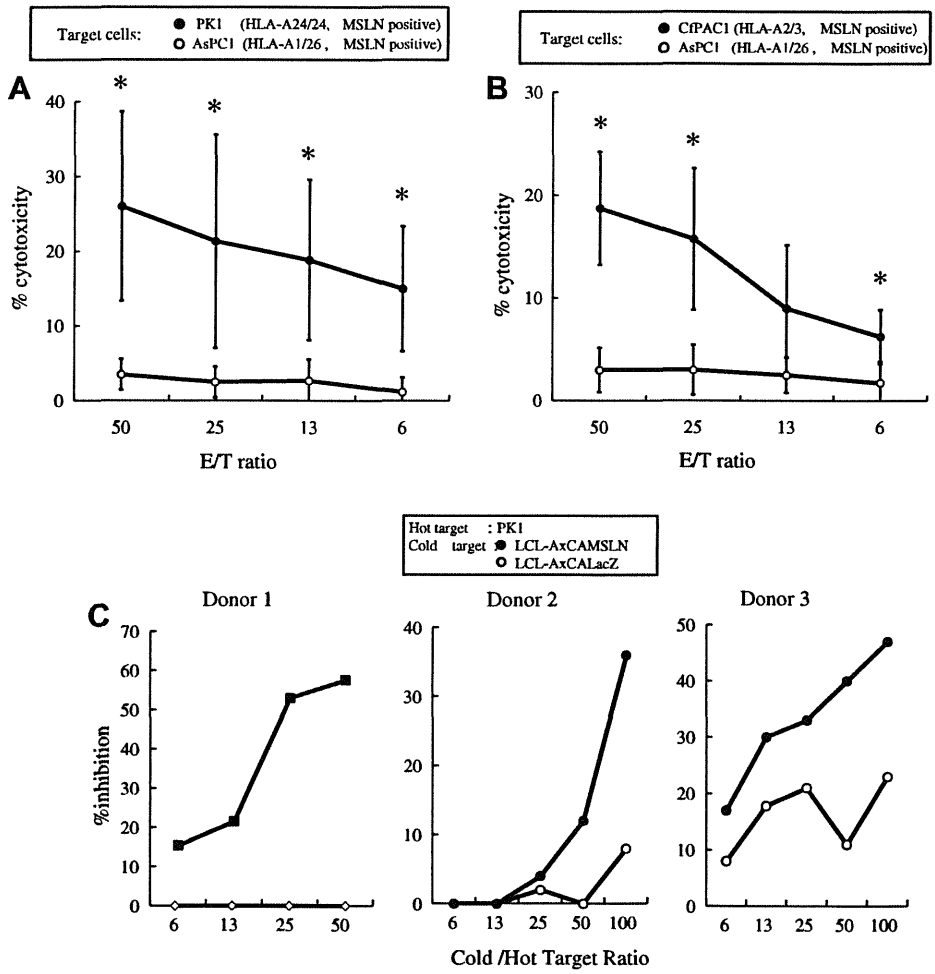

Fig. 3. Cytotoxic activity of CTLs generated from MSLN cDNA-transduced DCs against pancreatic cell lines endogenously expressing MSLN. **A.** Cytotoxic activity against PK1, which endogenously express MSLN and HLA-A24, and against AsPC1, which endogenously express MSLN but not HLA-A24. Results were shown as the mean ± SD of six different donors who possessed HLA-A24. *Significant increase of the lysis was shown ($p < 0.01$). **B.** Cytotoxic activity against CfPAC1, which endogenously express MSLN and HLA-A2, and against AsPC1, which endogenously express MSLN but not HLA-A2. Results were shown as the mean ± SD of four different donors who possessed HLA-A2. *Significant increase of the lysis was shown ($p < 0.05$) **C.** For the cold target inhibition assay, PK1 cells labeled with $Na_2{}^{51}CrO_4$ were prepared as the hot target, whereas MSLN-transduced autologous LCL were used as the cold target (inhibitor). The effector/target (E/T) ratio was fixed at 25.

LCL was examined. CTLs induced by DC-AxCAMSLN from an HLA-A24/A2-positive donor (Donor 1) showed specific lysis against LCL pulsed with MSLN peptides $A24_{(435-443)}$, $A24_{(475-483)}$, $A2_{(20-28)}$, and $A2_{(530-538)}$. CTLs induced by DC-AxCAMSLN from the HLA-A24/26-positive donor (Donor 2) exhibited specific lysis against LCL pulsed with MSLN peptides $A24_{(435-443)}$ and $A24_{(475-483)}$ (Fig. 5). These CTLs showed no cytotoxic activity against LCL that were not pulsed with the peptides.

4. Discussion

In the present study, we first found that CTLs induced by human DCs transduced with full-length MSLN cDNA had strong cytotoxic activity against not only autologous LCL transduced with the MSLN gene, but also pancreatic cancer cell lines naturally expressing MSLN in an HLA-restricted fashion.

In humans, MSLN has been demonstrated to be overexpressed in several cancer types, including pancreatic cancer, ovarian cancer, mesothelioma, lung cancer, uterine serous carcinoma and acute myeloid leukemia, although it is not expressed in normal tissues except mesothelial cells [11,12,14,30–35]. Argani et al. [11] found that MSLN staining was positive in all 60 resected of the primary pancreatic adenocarcinomas they examined, but was negative or only weakly expressed in adjacent normal pancreatic tissues. This finding has been confirmed by many other studies with microarrays, serial analysis of gene expression, and immunohistochemical staining [36–38]. MSLN was also confirmed to be overexpressed in resected invasive ductal adenocarcinomas and invasive carcinomas derived from IPMNs in the present study. Importantly, however, it was not expressed in adenomas and even in carcinoma in situ of IPMNs. Even in the same specimen, the expression of MSLN was observed in the invasive component of IPMNs, but not in the non-invasive components.

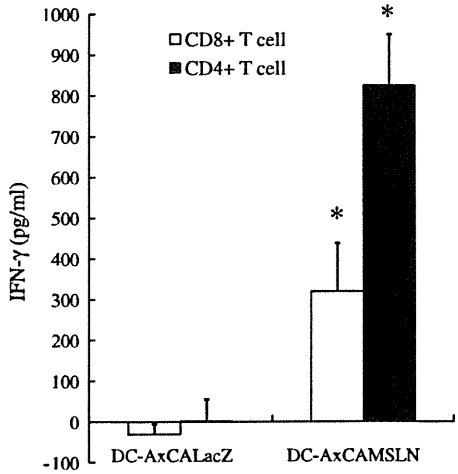

Fig. 4. MSLN-specific $CD4^+$ and $CD8^+$ T-cell responses induced by *in vitro* stimulation using MSLN cDNA-transduced DCs. $CD4^+$ and $CD8^+$ T cells were isolated using an autoMACS system from *in vitro* primed PBMCs after three cycles of re-stimulation by DC-AxCAMSLN cells. Each of the responding lymphocytes populations was stimulated with DC-AxCAMSLN or DC-AxCALacZ in a 96-well round-bottomed plate. Supernatants collected 24 h later were tested for IFN-γ levels. Results were shown as the mean ± SD of five different donors. *Significant increase of IFN-γ levels was shown ($p < 0.01$).

These results suggest that MSLN might have a tendency to be preferentially expressed in invasive tumor cells. In fact, MSLN plays a role in tumor adhesion and dissemination through the interaction between MSLN and MUC-16 [39,40]. MSLN also plays a role of cell proliferation and migration because these processes are inhibited by silencing of MSLN in pancreatic cancer cell lines [13,39]. In addition, normal mesothelial cells express no or little mesothelin protein [30,41,42], and therefore it is expected that MSLN-specific CTLs would not show the cytotoxic activity against them. MSLN is thus suggested to be an ideal target for immunotherapy for patients with pancreatic cancer because of its unique expression pattern, and because of its crucial functions that are closely related to malignant behavior.

With regard to the immunogenicity of MSLN, a clinical study conducted by Jaffee et al. that involved vaccination of pancreatic cancer patients with GM-CSF-transduced pancreatic cancer cell lines showed that 3 of 14 patients developed a post-vaccination delayed-type hypersensitivity (DTH) response to the autologous tumor that was associated with prolonged survival [43]. Interestingly, subsequent immunological studies showed that a strong induction of a $CD8^+$ T cell response to multiple HLA-restricted MSLN epitopes occurred in the 3 patients who had developed a vaccine-induced DTH response [28], thus suggesting that MSLN is strongly immunogenic. In addition, MSLN-specific $CD4^+$ and $CD8^+$ T cells were generated from peripheral lymphocytes of patients with pancreatic cancer in 50% of patients compared with only 20% of healthy individuals according to Johnston et al. [44]. Therefore, our vaccine strategy is expected to elicit MSLN-specific CTLs more effectively in patients with pancreatic cancer than in healthy individuals.

DCs are considered the most potent professional antigen-presenting cells and have the most powerful antigen presenting capacity [45,46]. Therefore, DCs adenovirally-transduced with full-length MSLN cDNA might have potential advantages over other cancer vaccine strategies. In this study, we demonstrated that CTLs induced by *in vitro* stimulation with DC-AxCAMSLN showed specific cytotoxic activity against not only autologous LCL transduced with MSLN cDNA, but also pancreatic cancer cell lines naturally expressing MSLN. In addition, CTLs induced by the stimulation with DC-AxCAMSLN showed specific cytotoxicity against autologous LCL pulsed with the multiple MSLN-derived epitope peptides. These results suggest that CTLs generated by the vaccine using DCs transduced with the full-length MSLN gene might have stronger cytotoxic

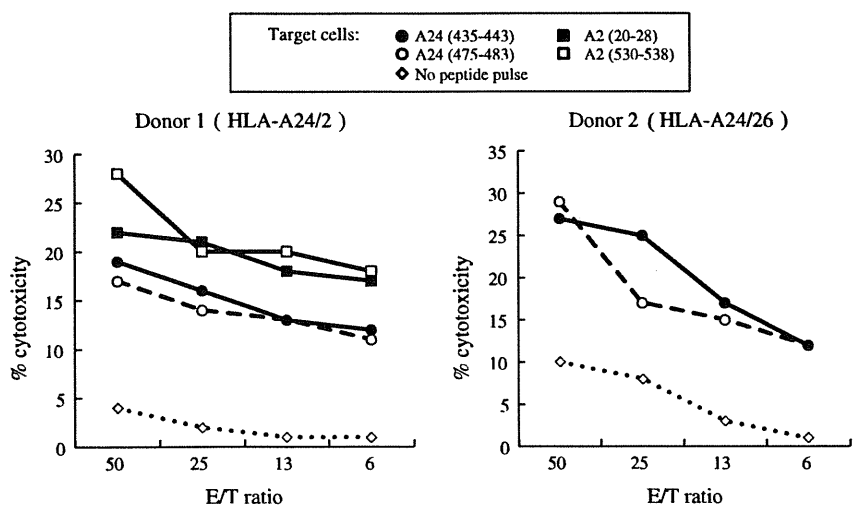

Fig. 5. The cytotoxic activity of CTLs generated from MSLN cDNA-transduced DCs against LCL pulsed with MSLN-epitope peptides. Donor 1 possessed HLA-A24/2 and Donor 2 possessed HLA-A24/26. The autologous LCL were pulsed with various epitope peptides derived from MSLN ($A24_{(435-443)}$, $A24_{(475-483)}$, $A2_{(20-28)}$, and $A2_{(530-538)}$), and were used as target cells.

activity than CTLs generated from one or two epitope peptides derived from MSLN-pulsed DCs, because they could recognize multiple epitopes and also unknown epitopes derived from MSLN.

It is generally accepted that the priming of anti-tumor $CD8^+$ CTLs requires $CD4^+$ T cells [47]. Our previous studies demonstrated that DCs transduced with the TAA gene could elicit tumor-specific $CD4^+$ T cells, and that those $CD4^+$ T cells played a critical role in the priming phase of $CD8^+$ CTLs because the anti-tumor effect was completely abrogated by the depletion of $CD4^+$ T cells in mouse models [23,24,48]. The present study also showed that DC-Ax-CAMSLN activated not only MSLN-specific $CD8^+$ T cells but also $CD4^+$ T cells by the IFN-γ release assay. Therefore, our DC vaccine strategy could more effectively induce MSLN-specific CTLs than the MSLN targeting used in previous studies that was based on single peptide [17,19,49].

In conclusion, MSLN is an ideal immunological target for pancreatic cancer in terms of its expression pattern, crucial cancer-related functions and immunogenicity. The *in vitro*-stimulation of PBMCs with DCs adenovirally-transduced with the entire MSLN gene elicited MSLN-specific cytotoxicity against pancreatic cancer cell lines by inducing the recognition of multiple MSLN epitopes, and activating both $CD8^+$ T cells and $CD4^+$ helper T cells. Therefore, vaccination using these genetically-modified DCs expressing the entire MSLN gene might be promising for clinical applications for patients with pancreatic cancer.

5. Conflicts of Interest

None declared.

Acknowledgements

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ORIGINAL ARTICLE

Influence of Visceral Obesity for Postoperative Pulmonary Complications After Pancreaticoduodenectomy

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Abstract
Background We conduct this study to determine whether postoperative complications, including postoperative pulmonary complications (PPCs), are associated with BMI and visceral fat area (VFA) after pancreaticoduodenectomy.
Methods A total of 317 patients undergoing pancreaticoduodenectomy were enrolled. VFA was measured using a cross-sectional computed tomography (CT) scan at the level of the umbilicus by FatScan software version 3.0 (N2 systems Inc., Osaka, Japan). Clinicopathological variables, intraoperative outcomes, and postoperative courses were analyzed.
Results Of all patients, 130 (41.0%) had postoperative complications and PPCs occurred in 14 patients (4.4%). VFA were significantly higher in patients who developed postoperative pancreatic fistula (POPF), PPCs, and mortality than in those patients who did not (P=.0282, P=.0058, and P=.0173, respectively). Multivariate analysis demonstrated that high BMI and high VFA were not independent predictive risk factors for POPF grade B/C and mortality; only high VFA was an independent risk factor influencing PPCs (P=.0390, odds ratio 4.246, 95% confidence interval 1.076–16.759).
Conclusions Visceral obesity was the independent risk factor for the incidence of PPCs after pancreaticoduodenectomy. Preoperative VFA measurement using CT scan is a useful tool for the prediction of the development of PPCs compared to BMI calculation.

Keywords Visceral fat area (VFA) · Body mass index (BMI) · Pancreaticoduodenectomy · Postoperative complications · Postoperative pulmonary complications (PPCs)

Introduction

The prevalence of overweight and obesity is increasing in the general population and reached over 60% in U.S. populations having higher than 25 kg/m^2 body mass index (BMI).[1] Overweight and obesity are associated with numerous complications such as cardiovascular, pulmonary, and metabolic disorders; therefore, surgeons have agreed that generalized obesity is a potential risk factor for operative morbidity and mortality.[2–5] Although BMI is a convenient measure and is useful for assessing the consequence of obesity, it is often unreliable for the evaluation of an individual's status because the proportion and distribution of fat tissue differ greatly from each other. Accordingly, in recent years, excessive visceral fat has been noteworthy in its association with postoperative complications.[6–8]

Postoperative pulmonary complications (PPCs) are common complications after all digestive surgery. Previous reports demonstrated that the incidence of PPCs was 2–13% after pancreaticoduodenectomy,[9–13] which was less than the incidence of postoperative pancreatic fistula (POPF), postpancreatectomy hemorrhage (PPH), and delayed gastric emptying (DGE).

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