Table 1 Baseline demographics

	Resectable	Borderline	*P*-value
Number of patients	582	389	–
Age (y), median (range)	68 (27–90)	65 (32–87)	<0.001
Sex (male : female)	320:262	229:160	0.23
Past medical history or Comorbid illness			
Hypertension, *n* (%)	166 (29)	92 (24)	0.091
Coronary arterial disease, *n* (%)	66 (11)	36 (11)	0.30
Diabetes mellitus, *n* (%)	175 (30)	120 (31)	0.80
Other malignancy, *n* (%)	94 (16)	41 (11)	0.012
Peptic ulcer, *n* (%)	45 (7.8)	25 (6.4)	0.44
Hepatitis, *n* (%)	39 (6.7)	20 (5.1)	0.31
Other digestive disease[a], *n* (%)	98 (17)	49 (13)	0.074
Respiratory disease, *n* (%)	41 (7.1)	20 (5.1)	0.22
Cerebral Vascular disease, *n* (%)	25 (4.3)	17 (4.4)	0.96
Jaundice, *n* (%)	190 (33)	169 (43)	<0.001
Biliary drainage, *n* (%)	187 (32)	169 (43)	<0.001
Pre-treatment serum tumor marker			
CEA (ng/ml), median (range)	3 (0–435)	3.5 (0–675)	0.0039
CA19-9 (U/ml), median (range)[b]	78 (0–47,470)	203.75 (0–220,540)	<0.001

[a] Other digestive disease included appendicitis ($n = 47$), cholecystolithiasis ($n = 33$), colonic polyps ($n = 16$), pancreatitis ($n = 10$), gastritis ($n = 8$), gastric polyps ($n = 5$), intestinal obstruction ($n = 4$), liver cirrhosis ($n = 3$), reflux esophagitis ($n = 2$), fatty liver ($n = 2$), hepatic hemangioma ($n = 2$), pancreatic cyst ($n = 2$), hemorrhoid ($n = 2$), irritable bowel disease, trauma, situs inversus, colonic derticulitis, ulcerative colitis, Peutz-Jeghers syndrome ($n = 1$), unknown ($n = 5$)

[b] The values of CA19-9 were measured after biliary drainage when the patients with jaundice

Table 2 Types of neoadjuvant therapy and agents

	Resectable	Borderline	*P*-value
Neoadjuvant therapy, *n* (%)	185 (32)	203 (52)	<0.0001
Types of therapy			
Radiotherapy, *n* (%)	114 (20)	140 (36)	<0.0001
Irradiation (Gy), median (range)	50 (35.2–54)	45 (10.8–67.5)	<0.0001
Agents provided			
With Gemcitabine	95	90	
With S1	8	28	
With Gemcitabine+S1	5	13	
With Other agents[a]	4	7	
Radiation alone	4	7	
Duration[b] (days), median (range)	99.5 (56–278)	82 (46–391)	<0.0001
Chemotherapy, *n* (%)	65 (11)	50 (13)	0.43
Agents provided			
Gemcitabine	17	22	
S1	5	2	
Gemcitabine+S1	26	24	
Gemcitabine+Other agents[c]	17	2	
Duration[b] (days), median (range)	28 (13–138)	81.5 (16–137)	0.0029
No record	6	13	

[a] Other agents was 5-FU + CDDP + MMC ($n = 11$)

[b] Duration represents the days from the start of neoadjuvant therapy to operation

[c] Other agents included 5-FU ($n = 18$) and CDDP ($n = 1$)

Table 3 Adverse events during neoadjuvant therapy

	Grade 1–4 (%)			Grade 3–4 (%)		
	Chemotherapy	Radiotherapy	*P*-value	Chemotherapy	Radiotherapy	*P*-value
Neutrocytopenia	58.8	65.4	0.30	33.8	20.0	0.0164
Leukocytopenia	53.8	75.5	0.0003	16.3	36.7	0.0007
Anemia	28.2	61.5	<0.0001	1.4	3.9	0.45
Thrombocytopenia	26.7	35.7	0.16	4.0	2.8	0.70
Fatigue	20.8	33.2	0.048	0.0	0.0	>0.99
Allergy	13.7	9.7	0.37	0.0	1.1	>0.99
Nausea/Vomiting	9.7	29.6	0.0006	1.4	2.2	>0.99
Liver dysfunction	3.1	0.4	0.055	0.0	0.0	>0.99
Pigmentation	2.3	1.8	0.70	0.0	0.0	>0.99
Anorexia	1.7	8.6	0.010	0.9	2.2	0.67
Cholangitis/Cholecystitis	0.8	2.5	0.43	0.0	2.1	0.17
Pneumonitis	0.0	2.5	0.094	0.0	1.3	0.56
Body weight loss	0.0	2.1	0.17	0.0	0.0	>0.99
Other[a]	6.1	2.5	0.93	0.0	0.0	>0.99

[a] Other non-hematological adverse events included thrombosis, peptic ulcer, oral mucositis, renal dysfunction, constipation

leukocytopenia occurred in more than half of the patients who received neoadjuvant therapy. Any grade leukocytopenia ($P = 0.0003$), anemia ($P < 0.0001$), fatigue ($P = 0.048$), nausea/vomiting ($P = 0.0006$), and anorexia ($P = 0.01$) were significantly more frequent in patients receiving chemoradiotherapy than systemic chemotherapy. Grade 3/4 neutropenia was significantly more frequent in patients receiving chemotherapy ($P = 0.0164$), whereas grade 3/4 leukocytopenia was significantly more frequent in patients receiving chemoradiotherapy ($P = 0.0007$). There were significant differences in any other AE.

Radiological tumor response to neoadjuvant therapy was assessed by tumor reduction rate, shown by waterfall chart analysis (Fig. 2). The median tumor reduction rate was 6.3% (range, –45.2–93.9%). According to Response Evaluation Criteria In Solid Tumors (RECIST) guidelines, 16% of patients showed a partial response, 80% had stable disease, and 4% had progressive disease (PD); none had a complete response to neoadjuvant therapy. Responses to neoadjuvant therapy were similar in patients with resectable and borderline resectable tumors ($P = 0.14$).

Resectability

Of the 388 patients who received neoadjuvant treatment, 25, including eight with resectable and 17 with borderline resectable tumors, did not undergo surgery, including 21 (84%) with PD and one with an AE during preoperative treatment. Of the 582 patients with resectable disease, 397 were scheduled for surgery-first, and, of these, 375 (94.5%) underwent resection. Similarly, of the 185 patients with resectable disease who received neoadjuvant therapy, 171 (92.4%) underwent resection ($P = 0.34$). R0 resection was performed on 305 patients in the surgery-first group and 164 in the neoadjuvant group. The R0 rate was significantly higher in the neoadjuvant than in the surgery-first group, both by on-treatment ($P < 0.0001$) and intention to treat ($P = 0.0003$) analysis (Table 4a). Of the 389 patients with borderline resectable disease, 186 were scheduled to undergo surgery first, and, of these, 156 (83.9%) underwent resection. Similarly, of the 203 patients with borderline resectable disease who received neoadjuvant treatment, 156 (77.8%) underwent resection ($P = 0.16$). Curability assessment showed no significant differences between the two groups, both by on-treatment and intention-to-treat analysis (Table 4b).

Perioperative outcomes

Perioperative morbidity and mortality were evaluated in the 870 patients who underwent pancreatic resection, after excluding the 76 patients who underwent exploratory or bypass surgery. Of these 870 patients, 16 (1.8%) died. In the 546 patients with resectable tumors, there were no significant differences between the neoadjuvant and surgery-first groups in the proportions that underwent various operative procedures or combined resection of major vessels. Operation time was significantly longer ($P = 0.0001$) and blood loss was significantly greater ($P = 0.0059$) in the neoadjuvant than in the surgery-first group. There were six operative deaths (1.6%) in the surgery-first group and one (0.6%) in the neoadjuvant group ($P = 0.44$). Median postoperative hospital stay was significantly longer

Table 4 Resection and R0-resection rate: (**a**) Resectable ($n = 582$) and (**b**) Borderline ($n = 389$)

Group	Surgery first	Neoadjuvant	*P*-value
(a) Resectable ($n = 582$)			
Total cohort, *n*	397	185	–
Resection, *n*	375	171	0.34
Resection rate	94.5%	92.4%	
R0 resection, *n*	305	164	
R0 rate by on-treatment analysis[a]	81.3%	95.9%	<0.0001
R0 rate by intention-to-treat analysis[b]	76.8%	88.6%	0.0003
(b) Borderline ($n = 389$)			
Total cohort	186	203	–
Resection	156	158	
Resection rate	83.9%	77.8%	0.16
R0 resection	118	123	
R0 rate by on-treatment analysis[a]	75.6%	77.8%	0.57
R0 rate by intention-to-treat analysis[b]	63.4%	60.6%	0.61

[a] R0 rate by on-treatment analysis was R0 resection per all resected cases with a record of residual tumor assessment

[b] R0 rate by intention-to-treat analysis was R0 resection per total cases with a record of residual tumor assessment including non-resected and non-operated cases as R2 resection

($P = 0.0020$), and morbidity rate was slightly but not significantly higher ($P = 0.084$) in the neoadjuvant than in the surgery-first group. There were no significant differences in specific postoperative complications, including pancreatic fistula and delayed gastric emptying, as well as in rates of severe complications and reoperation (Table 5).

Of the 314 patients who underwent resection for borderline resectable tumors, those who received neoadjuvant treatment were significantly more likely to undergo resection of the pancreas head ($P = 0.0026$) and portal vein ($P = 0.0018$) than those who underwent surgery first. Operation time was significantly longer in the neoadjuvant than in the surgery-first group ($P = 0.0005$), but there were no between group differences in blood loss ($P = 0.16$), mortality ($P = 0.17$), and hospital stay ($P = 0.50$) (Table 6). Morbidity tended to be less frequent in the neoadjuvant group than in the surgery-first group ($P = 0.057$). In contrast to patients with resectable tumors, the postoperative pancreatic fistula (POPF) rates in patients with borderline resectable tumors were significantly lower in the neoadjuvant group than in the surgery-first group, both for all grades ($P = 0.022$) and grade B/C ($P = 0.015$). Fluid collection was significantly more frequent in the neoadjuvant than in the surgery-first group ($P = 0.016$). Other specific complications and their severity were similar in these two groups (Table 6). In the resectable group with neoadjuvant therapy followed by resection, the proportion of delayed gastric emptying (DGE) in chemoradiotherapy was significantly higher than that in chemotherapy (21.6% vs. 10.1%, $P = 0.0015$). The proportion of other postoperative complications as well as severity of complications and reoperation listed in Table 5 was similar in both treatment modalities. In the borderline group with neoadjuvant therapy followed by resection, the proportion of grade B/C POPF in chemotherapy was slightly, but not statistically significant, higher than that in chemoradiotherapy (10.5% vs. 4.8%, $P = 0.092$). The proportion of other postoperative complications as well as severity of complications and reoperation listed in Table 6 was similar in both treatment modalities.

Histological staging

Table 7 shows a univariate comparison of histological staging according to the American Joint Committee on Cancer (AJCC). Of patients with resectable tumors, those who received neoadjuvant therapy had a lower T grade of the primary tumor than those who underwent surgery first ($P = 0.033$). Moreover, the percentage of patients with lymph node-positive tumors was significantly lower in the neoadjuvant than in the surgery-first group (30.6% vs. 55.2%, $P < 0.0001$), resulting in a significantly lower stage in the former ($P < 0.0001$). In patients with borderline resectable tumors, those who received neoadjuvant treatment had a significantly lower grade of the primary tumor ($P = 0.042$), a significantly lower rate of node-positive tumors (44.3% vs. 74.8%, $P < 0.0001$), and a significantly lower tumor stage ($P < 0.0001$).

Discussion

This survey clarified the feasibility, efficacy, and perioperative outcomes including resectability following

Table 5 Peri-operative outcome in resecteable group

Group	Surgery first	Neoadjuvant	*P*-value
Resection, *n*	375	171	–
PD, *n* (%)	236 (62.9)	111 (64.9)	0.66
DP, *n* (%)	126 (33.6)	52 (30.4)	0.46
TP, *n* (%)	12 (3.2)	7 (4.1)	0.60
PV resection, *n* (%)	71 (18.9)	37 (21.6)	0.46
Arterial resection, *n* (%)	4 (1.1)	4 (2.3)	0.27
Operative time (ml), median (range)	404 (141–829)	470 (157–1,021)	0.0001
Blood loss (ml), median (range)	872 (50–16,422)	1,088 (55–12,925)	0.0059
Blood transfusion (U), median (range)	2 (0–52)	2 (0–16)	0.65
Postoperative hospital stay (day), median (range)	31 (7–167)	36 (8–115)	0.0020
Morbidity, *n* (%)	194 (51.7)	102 (59.7)	0.084
POPF (all grade), *n* (%)	90 (24.0)	35 (20.5)	0.36
POPF (grade B/C), *n* (%)	43 (11.5)	20 (11.7)	0.94
DGE	40 (10.7)	27 (15.8)	0.10
Hemorrhage	16 (4.3)	7 (4.1)	0.93
Abscess	38 (10.1)	19 (11.1)	0.73
Wound infection	30 (8.0)	17 (9.9)	0.46
Leakage[a]	5 (1.3)	6 (3.5)	0.11
Pneumonitis	8 (2.1)	3 (1.8)	>0.99
Thrombosis	3 (0.8)	2 (1.2)	0.65
Cardiac disease	4 (1.0)	0 (0.0)	0.31
Brain	0 (0.0)	1 (0.6)	0.31
Fluid collection/	16 (4.3)	4 (2.3)	0.33
Hepatic disorder	4 (1.1)	4 (2.3)	0.27
Catheter infection	3 (0.8)	2 (1.2)	0.65
Ileus	4 (1.1)	1 (0.6)	>0.99
Cholangitis	4 (1.1)	0 (0.0)	0.31
Diarrhea/enteritis	10 (2.7)	6 (3.5)	0.59
DIC	2 (0.5)	1 (0.6)	>0.99
UTI	1 (0.3)	1 (0.6)	0.53
Renal disorder	2 (0.5)	0 (0.0)	>0.99
Anaphylaxis	1 (0.3)	1 (0.6)	0.53
Sepsis	1 (0.3)	0 (0.0)	>0.99
Splenic infarction	1 (0.3)	1 (0.6)	0.53
Peptic ulcer	1 (0.3)	0 (0.0)	>0.99
Herpes Zoster	2 (0.5)	0 (0.0)	>0.99
Portal vein trouble	1 (0.3)	0 (0.0)	>0.99
Severe complication (Grade IIIa–V), *n* (%)	58 (15.8)	23 (13.9)	0.59
Reoperation	9 (2.4)	7 (4.1)	0.29
Mortality, *n* (%)	6 (1.6)	1 (0.6)	0.44

[a] Leakage includes anastomosis insufficiency except for pancreatic fistula

neoadjuvant therapy in patients with pancreatic cancer. Adjuvant chemotherapy with gemcitabine is a standard therapy following resection for pancreatic cancer and significantly enhances recurrence-free and overall survival compared with surgery alone, with a median overall survival of almost 2 years after surgery [3–5]. However, this approach of surgery followed by adjuvant therapy cannot be offered to a significant proportion of patients with pancreatic cancer because of risks of surgical morbidity and the presence of unresectable disease at laparotomy. In contrast, almost all patients can receive neoadjuvant therapy before surgery [17, 18].

A major concern in treating these patients with neoadjuvant therapy is the risks of operative morbidity and

Table 6 Peri-operative outcome in borderline group

Group	Surgery first	Neoadjuvant	*P*-value
Resection, *n*	156	158	–
PD, *n* (%)	95 (60.9)	121 (76.6)	0.0026
DP, *n* (%)	51 (32.7)	31 (19.6)	0.0081
TP, *n* (%)	9 (5.8)	6 (3.8)	0.44
PV resection, *n* (%)	84 (53.9)	112 (70.9)	0.0018
Arterial resection, *n* (%)	13 (8.3)	10 (6.3)	0.50
Operative time (ml), median (range)	496 (161–1,221)	567 (190–1,160)	0.0005
Blood loss (ml), median (range)	1,137 (20–16,201)	1,400 (60–8,422)	0.16
Blood transfusion (U), median (range)	4 (0–54)	4 (0–18)	0.51
Postoperative hospital stay (day), median (range)	30 (7–397)	31 (8–124)	0.50
Morbidity, *n* (%)	93 (50.0)	82 (40.4)	0.057
POPF (all grade), *n* (%)	34 (18.3)	16 (7.9)	0.0022
POPF (gradeB/C), *n* (%)	19 (10.2)	8 (3.9)	0.015
DGE	24 (12.9)	20 (9.9)	0.34
Hemorrhage	3 (1.6)	4 (2.0)	0.55
Abscess	16 (8.6)	17 (6.4)	0.41
Wound infection	18 (9.7)	20 (9.9)	0.95
Leak[a]	8 (4.3)	3 (1.5)	0.13
Pneumonitis	2 (1.1)	4 (2.0)	0.69
Thrombosis	1 (0.5)	1 (0.5)	1.0
Cardiac disease	0 (0.0)	2 (1.0)	0.50
Brain	2 (1.1)	1 (0.5)	0.61
Fluid collection/	5 (2.7)	17 (8.4)	0.016
Hepatic disorder	3 (1.6)	5 (2.5)	0.73
Catheter infection	1 (0.5)	2 (1.0)	0.53
Ileus	1 (0.5)	0 (0.0)	0.48
Cholangitis	2 (1.1)	2 (1.0)	0.65
Diarrhea/enteritis	4 (2.2)	9 (4.4)	0.26
DIC	0 (0.0)	0 (0.0)	–
UTI	0 (0.0)	0 (0.0)	–
Renal disorder	0 (0.0)	0 (0.0)	–
Anaphylaxis	0 (0.0)	0 (0.0)	–
Splenic infarction	0 (0.0)	0 (0.0)	–
Peptic ulcer	1 (0.5)	1 (0.5)	1.0
Herpes Zoster	0 (0.0)	0 (0.0)	–
Portal vein trouble	1 (0.5)	1 (0.5)	1.0
Severe complication (Grade IIIa–V), *n* (%)	22 (14.5)	21 (13.7)	0.85
Reoperation	6 (3.9)	6 (3.8)	0.98
Mortality, *n* (%)	2 (1.3)	7 (4.4)	0.17

[a] Leakage includes anastomosis insufficiency except for pancreatic fistula

mortality. Although several small prospective studies have demonstrated the feasibility of this approach [10, 11, 19], this has not been confirmed because of the small sample sizes. Several nationwide surveys [20, 21] and systematic reviews and meta-analyses [22, 23] indicated that this strategy was feasible in larger numbers of patients, but could not quantify the data. Only one systematic review showed the rate of surgical morbidity and mortality after neoadjuvant therapy [24]. We found that neoadjuvant treatment did not significantly increase perioperative mortality and morbidity rates, including pancreatic fistula and delayed gastric emptying, indicating that neoadjuvant treatment was a feasible strategy in patients with pancreatic cancer. Neoadjuvant therapy, however, resulted in significantly longer operation times and postoperative hospital stay, as well as higher rates of grade 3/4 hematological toxicities. Nevertheless, these preoperative toxicities were manageable, with <0.5% of patients becoming ineligible for surgery.

Table 7 Peri-operative outcome in resecteable group: (**a**) Resectable and (**b**) Borderline

		Surgery first	Neoadjuvant	*P*-value
(a) Resectable				
T	0	1 (0.3)	2 (1.2)	0.033
	1	33 (8.8)	28 (16.5)	
	2	35 (9.3)	13 (7.7)	
	3	304 (81.1)	124 (72.9)	
	4	2 (0.5)	3 (1.8)	
N	0	168 (44.8)	118 (69.4)	<0.0001
	1	207 (55.2)	52 (30.6)	
M	0	354 (94.4)	160 (94.1)	0.895
	1	21 (5.6)	10 (5.9)	
Stage	0	0 (0)	2 (0.4)	<0.0001
	IA	28 (7.5)	24 (14.1)	
	IB	28 (7.5)	10 (5.9)	
	IIA	110 (29.3)	81 (47.7)	
	IIB	186 (49.6)	40 (23.5)	
	III	2 (0.5)	3 (1.7)	
	IV	21 (5.6)	10 (5.9)	
(b) Borderline				
T	0	2 (1.3)	1 (0.6)	0.042
	1	2 (1.3)	12 (7.6)	
	2	4 (2.6)	9 (5.7)	
	3	140 (90.3)	129 (82.2)	
	4	7 (4.5)	6 (3.8)	
N	0	39 (25.2)	88 (55.7)	<0.0001
	1	116 (74.8)	70 (44.3)	
M	0	132 (85.2)	143 (90.5)	0.895
	1	23 (14.8)	15 (9.5)	
Stage	0	0 (0.0)	1 (0.6)	<0.0001
	IA	1 (0.7)	10 (6.3)	
	IB	2 (1.3)	5 (3.2)	
	IIA	32 (20.7)	64 (40.5)	
	IIB	91 (58.7)	58 (36.7)	
	III	6 (3.9)	5 (3.2)	
	IV	23 (14.8)	15 (9.5)	

Another concern associated with the neoadjuvant strategy is a possible decrease in tumor resectability due to tumor progression during preoperative treatment. A meta-analysis showed that, of patients with resectable tumors, 73.6% to 82.9% remained resectable after neoadjuvant therapy [17, 24], findings similar to those in patients scheduled for primary resection and adjuvant therapy. We found that neoadjuvant therapy did not decrease tumor resectability, both in patients with resectable and borderline resectable pancreatic cancers. Intention-to-treat analysis showed that, in resectable tumors, the curability (R0 resection rate) was improved after neoadjuvant treatment. Radiologically, 90% of patients who received neoadjuvant therapy showed lack of tumor progression or tumor shrinkage, with only 10% showing tumor progression, suggesting that neoadjuvant treatment increased the likelihood of curative resection. These advantages of neoadjuvant therapy, however, were not observed in patients with borderline resectable disease, and resectability and R0 resectability were similar in the neoadjuvant and surgery-first groups. The incidence of nodal involvement was significantly lower in the neoadjuvant than in the surgery-first group. Neoadjuvant therapy has been reported to reduce the number of lymph node metastases [25, 26], suggesting that the main effect of neoadjuvant therapy is to reduce peripancreatic lymph node positivity rather than the size of primary tumors. Since nodal involvement is one of the most significant predictors of patient survival [27, 28], neoadjuvant therapy may have a survival benefit following resection of pancreatic cancer.

Although the number of patients receiving neoadjuvant therapy is the largest to date, questionnaire surveys have limitations. Data were collected from the various treatment centers retrospectively, not prospectively. In addition, there was significant inter-center heterogeneity in eligibility criteria for neoadjuvant treatment, neoadjuvant regimens, radiologic and intraoperative indications for resection, and postoperative therapy regimens. This heterogeneity may have introduced selection biases, preventing definite conclusions. Prospectively designed trials with adequate numbers of patients are required to determine the feasibility and efficacy of neoadjuvant treatment in patients with pancreatic cancer. This survey analyzing the effects of neoadjuvant treatment on resectablity and perioperative outcomes in patients with pancreatic cancer could not determine the impact of treatment on survival. However, several studies have reported that neoadjuvant therapy had survival benefits in patients with resectable or borderline resectable pancreatic cancer [11, 17, 21, 22, 24]. These suggest the need for prospective randomized studies to clarify the effects on survival of neoadjuvant therapy compared with the standard surgery-first strategy, in patients with pancreatic cancer [12, 18]. In conclusion neoadjuvant therapy may not increase the mortality and morbidity rates, and may be able to increase the chance for curative resection especially against resectable tumor.

Acknowledgment This work was supported in part by Grants-in-Aid for Scientific Research (C) 21591766 from the Japan Society for the Promotion of Science.

Conflict of interest None declared.

References

1. Neoptolemos JP, Dunn JA, Stocken DD, Almond J, Link K, Beger H, et al.; European Study Group for Pancreatic Cancer. Adjuvant

chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. Lancet. 2001;358:1576–85.
2. Stocken DD, Büchler MW, Dervenis C, Bassi C, Jeekel H, Klinkenbijl JH, et al.; Pancreatic Cancer Meta-analysis Group. Meta-analysis of randomised adjuvant therapy trials for pancreatic cancer. Br J Cancer. 2005;92:1372–81.
3. Oettle H, Post S, Neuhaus P, Gellert K, Langrehr J, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. JAMA. 2007;297:267–77.
4. Ueno H, Kosuge T, Matsuyama Y, Yamamoto J, Nakao A, Egawa S, et al. A randomised phase III trial comparing gemcitabine with surgery-only in patients with resected pancreatic cancer: Japanese Study Group of Adjuvant Therapy for Pancreatic Cancer. Br J Cancer. 2009;101:908–15.
5. Neoptolemos JP, Stocken DD, Bassi C, Ghaneh P, Cunningham D, Goldstein D, et al.; European Study Group for Pancreatic Cancer. Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial. JAMA. 2010;304:1073–81.
6. Kaufmann M, Morrow M, von Minckwitz G, Harris JR, Biedenkopf Expert Panel Members. Locoregional treatment of primary breast cancer: consensus recommendations from an International Expert Panel. Cancer. 2010;116:1184–91.
7. Ando N, Kato H, Igaki H, Shinoda M, Ozawa S, Shimizu H, et al. A randomized trial comparing postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil versus preoperative chemotherapy for localized advanced squamous cell carcinoma of the thoracic esophagus (JCOG9907). Ann Surg Oncol. 2012;19:68–74.
8. Palmer DH, Stocken DD, Hewitt H, Markham CE, Hassan AB, Johnson PJ, et al. A randomized phase 2 trial of neoadjuvant chemotherapy in resectable pancreatic cancer: gemcitabine alone versus gemcitabine combined with cisplatin. Ann Surg Oncol. 2007;14:2088–96.
9. Heinrich S, Pestalozzi BC, Schäfer M, Weber A, Bauerfeind P, Knuth A, et al. Prospective phase II trial of neoadjuvant chemotherapy with gemcitabine and cisplatin for resectable adenocarcinoma of the pancreatic head. J Clin Oncol. 2008; 26:2526–31.
10. Varadhachary GR, Wolff RA, Crane CH, Sun CC, Lee JE, Pisters PW, et al. Preoperative gemcitabine and cisplatin followed by gemcitabine-based chemoradiation for resectable adenocarcinoma of the pancreatic head. J Clin Oncol. 2008;26:3487–95.
11. Evans DB, Varadhachary GR, Crane CH, Sun CC, Lee JE, Pisters PW, et al. Preoperative gemcitabine-based chemoradiation for patients with resectable adenocarcinoma of the pancreatic head. J Clin Oncol. 2008;26:3496–502.
12. Reni M. Neoadjuvant treatment for resectable pancreatic cancer: time for phase III testing? World J Gastroenterol. 2010;16:4883–7.
13. National Comprehensive Cancer Network. NCCN clinical practice guidelines in oncology. Pancreatic adenocarcinoma, version 2.2010. http://www.nccn.org/professionals/physician_gls/f_guidelines.asp
14. Cancer Therapy Evaluation Program. Common terminology criteria for adverse events, Version 3.0. http://ctep.cancer.gov
15. Bassi C, Dervenis C, Butturini G, Fingerhut A, Yeo C, Izbicki J, et al.; International Study Group on Pancreatic Fistula Definition. Postoperative pancreatic fistula: an international study group (ISGPF) definition. Surgery. 2005;138:8–13.
16. Wente MN, Bassi C, Dervenis C, Fingerhut A, Gouma DJ, Izbicki JR, et al. Delayed gastric emptying (DGE) after pancreatic surgery: a suggested definition by the International Study Group of Pancreatic Surgery (ISGPS). Surgery. 2007;142:761–8.
17. Andriulli A, Festa V, Botteri E, Valvano MR, Koch M, Bassi C, et al. Neoadjuvant/preoperative gemcitabine for patients with localized pancreatic cancer: a meta-analysis of prospective studies. Ann Surg Oncol. 2012;19:1644–62.
18. Heinrich S, Pestalozzi B, Lesurtel M, Berrevoet F, Laurent S, Delpero JR, et al. Adjuvant gemcitabine versus NEOadjuvant gemcitabine/oxaliplatin plus adjuvant gemcitabine in resectable pancreatic cancer: a randomized multicenter phase III study (NEOPAC study). BMC Cancer. 2011;11:346.
19. Le Scodan R, Mornex F, Girard N, Mercier C, Valette PJ, Ychou M, et al. Preoperative chemoradiation in potentially resectable pancreatic adenocarcinoma: feasibility, treatment effect evaluation and prognostic factors, analysis of the SFRO-FFCD 9704 trial and literature review. Ann Oncol. 2009;20:1387–96.
20. Stessin AM, Meyer JE, Sherr DL. Neoadjuvant radiation is associated with improved survival in patients with resectable pancreatic cancer: an analysis of data from the surveillance, epidemiology, and end results (SEER) registry. Int J Radiat Oncol Biol Phys. 2008;72:1128–33.
21. Sata N, Kurashina K, Nagai H, Nagakawa T, Ishikawa O, Ohta T, et al. The effect of adjuvant and neoadjuvant chemo(radio)therapy on survival in 1,679 resected pancreatic carcinoma cases in Japan: report of the national survey in the 34th annual meeting of Japanese Society of Pancreatic Surgery. J Hepatobiliary Pancreat Surg. 2009;16:485–92.
22. Artinyan A, Anaya DA, McKenzie S, Ellenhorn JD, Kim J. Neoadjuvant therapy is associated with improved survival in resectable pancreatic adenocarcinoma. Cancer. 2011;117: 2044–9.
23. Laurence JM, Tran PD, Morarji K, Eslick GD, Lam VW, Sandroussi C. A systematic review and meta-analysis of survival and surgical outcomes following neoadjuvant chemoradiotherapy for pancreatic cancer. J Gastrointest Surg. 2011;15:2059–69.
24. Gillen S, Schuster T, Meyer zum Buschenfelde C, Friess H, Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med. 2010;7:e1000267.
25. Ohigashi H, Ishikawa O, Eguchi H, Takahashi H, Gotoh K, Yamada T, et al. Feasibility and efficacy of combination therapy with preoperative full-dose gemcitabine, concurrent three-dimensional conformal radiation, surgery, and postoperative liver perfusion chemotherapy for T3-pancreatic cancer. Ann Surg. 2009;250:88–95.
26. Satoi S, Toyokawa H, Yanagimoto H, Yamamoto T, Kamata M, Ohe C, et al. Neo-adjuvant chemoradiation therapy using S-1 followed by surgical resection in patients with pancreatic cancer. J Gastrointest Surg. 2012;16:784–92.
27. Matsuno S, Egawa S, Fukuyama S, Motoi F, Sunamura M, Isaji S, et al. Pancreatic Cancer Registry in Japan: 20 years of experience. Pancreas. 2004;28:219–30.
28. Motoi F, Rikiyama T, Katayose Y, Egawa S, Unno M. Retrospective evaluation of the influence of postoperative tumor marker status on survival and patterns of recurrence after surgery for pancreatic cancer based on RECIST guidelines. Ann Surg Oncol. 2011;18:371–9.

OPEN ACCESS Freely available online

PLOS | ONE

Identification of an HLA-A2-Restricted Epitope Peptide Derived from Hypoxia-Inducible Protein 2 (HIG2)

Sachiko Yoshimura[1,2], Takuya Tsunoda[1,2,3], Ryuji Osawa[1,2], Makiko Harada[2], Tomohisa Watanabe[2], Tetsuro Hikichi[2], Masahiro Katsuda[1], Motoki Miyazawa[1], Masaji Tani[1], Makoto Iwahashi[1], Kazuyoshi Takeda[4], Toyomasa Katagiri[3,5], Yusuke Nakamura[3,6], Hiroki Yamaue[1]*

1 Second Department of Surgery, Wakayama Medical University, Wakayama, Japan, **2** OncoTherapy Science Inc, Research and Development Division, Kanagawa, Japan, **3** Laboratory of Molecular Medicine Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **4** Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan, **5** Division of Genome Medicine, Institute for Genome Research, The University of Tokushima, Tokushima, Japan, **6** Department of Medicine, University of Chicago, Chicago, Illinois, United States of America

Abstract

We herein report the identification of an HLA-A2 supertype-restricted epitope peptide derived from hypoxia-inducible protein 2 (HIG2), which is known to be a diagnostic marker and a potential therapeutic target for renal cell carcinoma. Among several candidate peptides predicted by the HLA-binding prediction algorithm, HIG2-9-4 peptide (VLNLYLLGV) was able to effectively induce peptide-specific cytotoxic T lymphocytes (CTLs). The established HIG2-9-4 peptide-specific CTL clone produced interferon-γ (IFN-γ) in response to HIG2-9-4 peptide-pulsed HLA-A*02:01-positive cells, as well as to cells in which HLA-A*02:01 and HIG2 were exogenously introduced. Moreover, the HIG2-9-4 peptide-specific CTL clone exerted cytotoxic activity against HIG2-expressing HLA-A*02:01-positive renal cancer cells, thus suggesting that the HIG2-9-4 peptide is naturally presented on HLA-A*02:01 of HIG-2-expressing cancer cells and is recognized by CTLs. Furthermore, we found that the HIG2-9-4 peptide could also induce CTLs under HLA-A*02:06 restriction. Taken together, these findings indicate that the HIG2-9-4 peptide is a novel HLA-A2 supertype-restricted epitope peptide that could be useful for peptide-based immunotherapy against cancer cells with HIG2 expression.

Citation: Yoshimura S, Tsunoda T, Osawa R, Harada M, Watanabe T, et al. (2014) Identification of an HLA-A2-Restricted Epitope Peptide Derived from Hypoxia-Inducible Protein 2 (HIG2). PLoS ONE 9(1): e85267. doi:10.1371/journal.pone.0085267

Editor: Rachel Louise Allen, University of London, St George's, United Kingdom

Received September 20, 2013; **Accepted** November 25, 2013; **Published** January 8, 2014

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yamaue-h@wakayama-med.ac.jp

Introduction

Renal cell carcinoma (RCC) comprises approximately 2–3% of all human malignancies [1]. Although patients with localized RCC can be curable by radical nephrectomy, approximately 30% of patients are observed to have metastasis at the time of diagnosis, and the median survival is only 1.5 years. Furthermore, 30% of patients experience a relapse after initial surgery, and no adjuvant treatment has yet been established [2–4]. Several molecular targeting agents, including the recently approved VEGFR tyrosine kinase inhibitor [5], were developed as novel therapeutics for RCC, but the majority of patients eventually develop treatment-resistant disease [6–13]. It is notable that RCC is one of the most immune responsive cancers. IL-2 based immunotherapy is currently the only curative treatment for metastatic RCC, but it is poorly tolerated, with significant side effects, and the efficacy has been limited to a 20% response rate, including a 5–10% complete response rate [14–17]. This limited success poses further challenges to improve the efficacy of immunotherapies for RCC. While therapeutic vaccines that induce immunity in response to tumor antigens have been under investigation for decades, the number of antigens identified in RCC and the efficacy in clinical trials have been limited [18–21].

Hypoxia-inducible protein 2 (*HIG2*) was first annotated as a novel gene induced by hypoxia and glucose deprivation [22]. A recent functional analysis revealed that HIG2 is a novel lipid droplet protein that stimulates intracellular lipid accumulation [23]. We reported HIG2 upregulation in RCC, and suggested its usefulness as a diagnostic biomarker for RCC [24]. Our findings also implied that HIG2 might be a good molecular target for the development of novel cancer treatment, because its expression was hardly detectable in normal organs except for the fetal kidney. Importantly, significant growth suppression of RCC cells occurred when endogenous *HIG2* was suppressed by *HIG2*-specific RNAi, suggesting that HIG2 has an essential role in the proliferation of RCC cells. An additional study revealed that HIG2 expression was found in 86% of human RCC tissue samples (80/93) and also correlated with the clinicopathological characteristics and survival of RCC patients [25].

In the present study, we focused on HIG2 as a novel tumor antigen, which induces antigen-specific cytotoxic T lymphocytes (CTLs) against RCC cells. We investigated the HIG2-derived epitope peptide restricted to HLA-A*02:01, the most common HLA class I type in Caucasians and the second most common type in the Japanese population [26,27], and demonstrate that this epitope peptide can also be presented by another HLA-A2 supertype allele. Thus, this epitope peptide would be applicable for peptide-based immunotherapies for RCC patients with HLA-A2.

Ethics statement

The study protocol was approved by the Institutional Review Board of OncoTherapy Science, Inc. and written informed consent was obtained from all subjects, in accordance with the guidelines of the Ethical Committee on Human Research of Wakayama Medical University, School of Medicine, OncoTherapy Science, Inc., The University of Tokyo, Juntendo University School of Medicine, The University of Tokushima and University of Chicago.

Materials and Methods

Peptides

HIG2-derived 9-mer and 10-mer peptides that have high binding affinity (binding score >10) to HLA-A:*02:01 were predicted by the binding prediction software "BIMAS" (http://www-bimas.cit.nih.gov/molbio/hla_bind), and the homologous sequences were examined by the homology search program "BLAST" (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Selected high affinity peptides and the HLA-A*02:01-restricted HIV-derived epitope peptide (ILKEPVHGV) [28] were synthesized by Sigma (Ishikari, Japan). The purity (>90%) and the sequences of the peptides were confirmed by analytical HPLC and a mass spectrometry analysis, respectively. Peptides were dissolved in dimethylsulfoxide at 20 mg/ml and stored at −80°C.

Cell lines

T2 (HLA-A*02:01, lymphoblast), Jiyoye (HLA-A32, Burkitt's lymphoma), EB-3 (HLA-A3/Aw32, Burkitt's lymphoma), *Cercopithecus aethiops*-derived COS7 and A498 (HLA-A*02:01, kidney carcinoma) cells were purchased from the American Type Culture Collection (Rockville, MD). PSCCA0922 (HLA-A*02:06/A*31:01, a B cell line) was provided by the Health Science Research Resources Bank (Osaka, Japan). Caki-1 (HLA-A*24:02/A*23:01, renal clear cell carcinoma) cells were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University. The HIG2 expression in A498 and Caki-1 cells was confirmed by a Western blotting analysis [24]. T2, Jiyoye, EB-3 and PSCCA0922 cells were maintained in RPMI1640 (Invitrogen, Carlsbad, CA), A498 and Caki-1 cells were maintained in EMEM (Invitrogen) and COS7 cells were maintained in DMEM (Invitrogen). Each medium was supplemented with 10% fetal bovine serum (GEMINI Bio-Products, West Sacramento, CA) and 1% antibiotic solution (Sigma-Aldrich, ST. Louis, MO).

Gene transfection

The plasmid encoding *HLA-A*02:01* was a generous gift from Dr. Kawakami (Keio University, Tokyo Japan). cDNA fragments encoding *HLA-A*02:06* or *HIG2* (GenBank Accession Number NM_013332) were cloned into the pcDNA3.1/myc-His vector (Invitrogen). Plasmid DNAs containing *HLA-A*02:01*, *HLA-A*02:06* and/or *HIG2* were transfected into COS7 cells using Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. COS7 cells were incubated with the transfection mixture at 37°C overnight prior to use as stimulator cells. The introduction of the targeted proteins was confirmed by a Western blotting analysis.

In vitro CTL induction

$CD8^+$ T cells and monocyte-derived dendritic cells (DCs) were prepared from peripheral blood of healthy volunteers (either HLA-A*02:01 or HLA-A*02:06 positive) with written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and $CD8^+$ T cells were harvested by positive selection with a Dynal CD8 Positive Isolation Kit (Invitrogen). Monocytes were enriched from the $CD8^-$ cell population by adherence to a tissue culture dish (Becton Dickinson, Franklin Lakes, NJ) and were cultured in AIM-V (Invitrogen) containing 2% heat-inactivated autologous serum (AS), 1,000 U/ml of GM-CSF (R&D Systems, Minneapolis, MN) and 1,000 U/ml of interleukin (IL)-4 (R&D Systems) on day 1. On day 4, 0.1 KE/ml of OK-432 (Chugai Pharmaceutical Co., Tokyo, Japan) was added in the culture to induce the maturation of DCs. On day 7, DCs were pulsed with 20 μg/ml of the respective synthesized peptides in the presence of 3 μg/ml of β2-microglobulin (Sigma-Aldrich, ST. Louis, MO) in AIM-V at 37°C for 4 h [29]. These peptide-pulsed DCs were then incubated with 30 μg/ml of mitomycin C (MMC) (Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) at 37°C for 30 min. Following washing out the residual peptide and MMC, DCs were cultured with autologous $CD8^+$ T cells on 48 well plates (Corning, Inc., Corning, NY) (each well contained 1.5×10^4 peptide-pulsed DCs, 3×10^5 $CD8^+$ T cells and 10 ng/ml of IL-7 (R&D Systems) in 0.5 ml of AIM-V/2% AS). Two days later, these cultures were supplemented with IL-2 (CHIRON, Emeryville, CA) (final concentration: 20 IU/ml). On days 14 and 21, T cells were further re-stimulated with the autologous peptide-pulsed DCs, which were freshly prepared every time. On day 28, the CTL activity against peptide-pulsed T2 or PSCCA0922 cells was examined by an interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assay.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

The human IFN-γ ELISPOT kit and AEC substrate set (BD Biosciences) were used to analyze the T cell response to the respective peptides. The ELISPOT assay was performed according to the manufacturer's instructions. Briefly, T2 or PSCCA0922 cells were pulsed with 20 μg/ml of the respective peptides at 37°C for 20 h, and the residual peptide that did not bind to cells was washed out to prepare peptide-pulsed cells as the stimulator cells. After removing 500 μl of supernatant from each well of *in vitro* CTL-inducing cultures, 200 μl of cell culture suspensions were harvested from each well and distributed to two new wells (100 μl each) on Multiscreen-IP 96 well plates (Millipore, Bedford, MA). The cells were co-incubated with peptide-pulsed cells (1×10^4 cells/well) at 37°C for 20 h. HIV peptide-pulsed cells were used as a negative control. Spots were captured and analyzed by an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology Ltd, Shaker Heights, OH) and the ImmunoSpot Professional Software package, Version 5.0 (Cellular Technology Ltd).

CTL expanding culture

The peptide-specific CTLs harvested from ELISPOT-positive wells after *in vitro* CTL induction were expanded by a modified protocol based on the previously described methods [30,31]. A total of 5×10^4 CTLs was cultured with 5×10^6 MMC-inactivated Jiyoye or EB-3 cells (30 μg/ml at 37°C for 30 min treatment) in 25 ml of AIM-V/5% AS containing 40 ng/ml of anti-CD3 monoclonal antibody (BD Biosciences, San Diego, CA) on day 0. IL-2 was added 24 h later (final concentration: 120 IU/ml), and fresh AIM-V/5% AS containing 30 IU/ml of IL-2 was provided on days 5, 8 and 11. On day 14, CTLs were harvested and the CTL activity was examined by an IFN-γ enzyme-linked immunosorbent assay (ELISA).

Establishment of CTL clones

CTL clones were established by the limiting dilution method. Briefly, CTLs were diluted to 0.3, 1 or 3 cells per well in 96 well

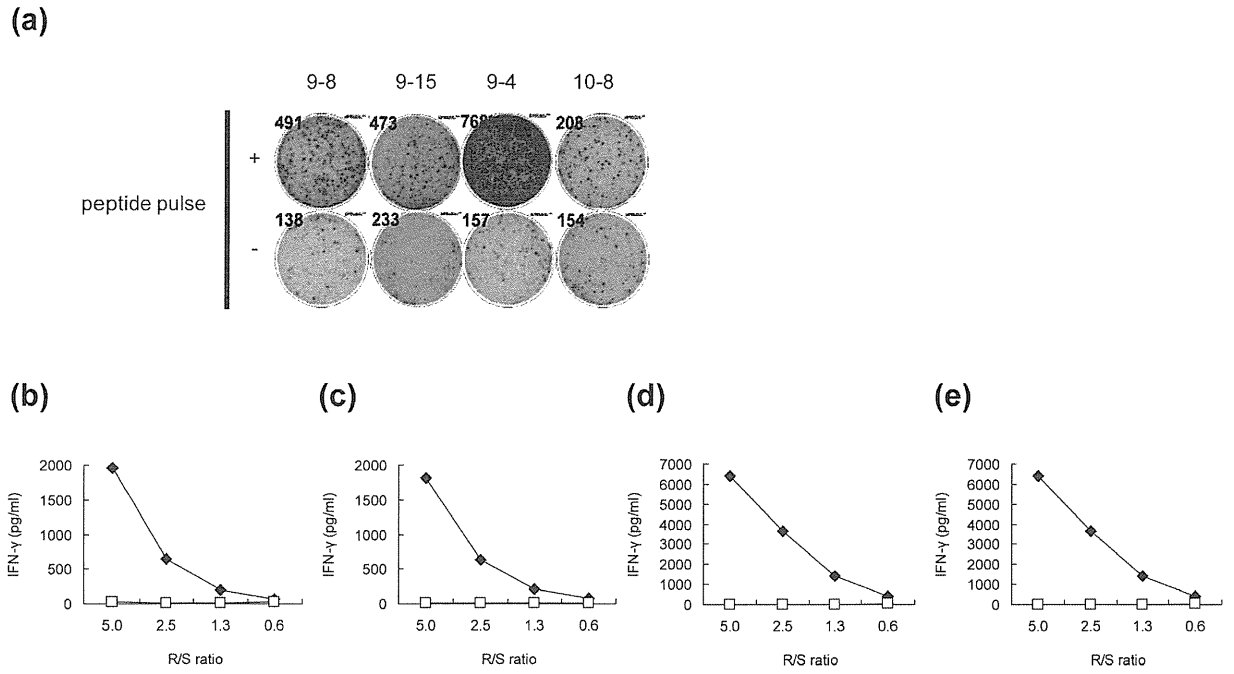

Figure 1. The IFN-γ production in response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide. (a) The IFN-γ production from cells induced by the indicated peptide-pulsed DCs was examined by an ELISPOT assay using T2 cells. "+" indicates the wells in which cells were stimulated with T2 cells pulsed with the indicated peptide and "−" indicates the wells in which cells were stimulated with HIV peptide-pulsed T2 cells. The IFN-γ production from cells induced with HIG2-9-8 (b), HIG2-9-15 (c), HIG2-9-4 (d) or HIG2-10-8 (e) peptide stimulation after CTL expanding culture was examined by ELISA. Cells were stimulated with T2 cells pulsed with the corresponding peptide (closed diamonds) or HIV peptide (open squares) at the indicated responder/stimulator ratio (R/S ratio). Similar results were obtained from three independent experiments.
doi:10.1371/journal.pone.0085267.g001

round bottom plates (Corning, Inc.), and were cultured with MMC-treated 1×10^4 Jiyoye and EB-3 cells in 125 μl AIM-V containing 5% AB serum and 30 ng/ml of an anti-CD3 monoclonal antibody on day 0. IL-2 was added to each well on day 10 (final concentration: 125 IU/ml). On day 14, an IFN-γ ELISPOT assay was performed to measure the CTL activity of each clone.

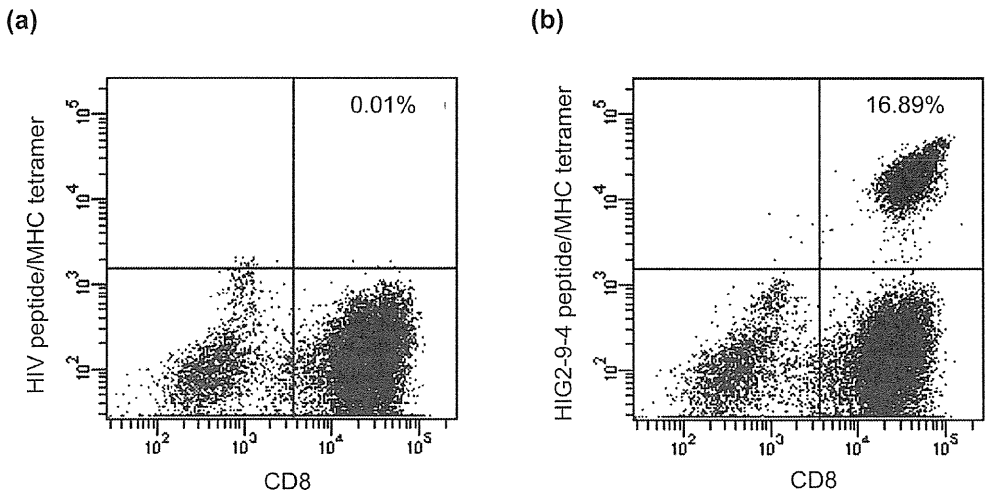

Figure 2. The expression of a HIG2-9-4 peptide-specific T cell receptor on CD8+ T cells. The expression of the HIG2-9-4 peptide-specific T cell receptor was examined on $CD3^+CD4^-$ cells following CTL expansion culture of HIG2-9-4 peptide-induced CTLs. (a) A quadrant gate was set based on the staining results with the HIV peptide/HLA-A*02: 01 tetramer. (b) $CD8^+$ T cells expressing the HIG2-9-4 peptide/HLA-A*02: 01-specific T cell receptor were detected. Similar results were obtained from three independent experiments.
doi:10.1371/journal.pone.0085267.g002

Table 1. Candidate peptides derived from HIG2 restricted with HLA-A*02:01.

Peptide name	Amino acid sequence (mer)	Binding Score
HIG2-9-8	YLLGVVLTL (9)	836.253
HIG2-9-13	VLTLLSIFV (9)	650.311
HIG2-9-15	TLLSIFVRV (9)	488.951
HIG2-9-4	VLNLYLLGV (9)	271.948
HIG2-9-9	LLGVVLTLL (9)	83.527
HIG2-9-22	RVMESLEGL (9)	31.957
HIG2-9-6	NLYLLGVVL (9)	28.027
HIG2-10-8	YLLGVVLTLL (10)	836.253
HIG2-10-29	GLLESPSPGT (10)	113.047
HIG2-10-4	VLNLYLLGVV (10)	14.495
HIG2-10-15	TLLSIFVRVM (10)	13.174
HIG2-10-18	SIFVRVMESL (10)	12.248

The binding score was obtained from the BIMAS website (http://www-bimas.cit.nih.gov/molbio/hla_bind).
doi:10.1371/journal.pone.0085267.t001

IFN-γ enzyme-linked immunosorbent assay (ELISA)

The CTL activity was examined by IFN-γ ELISA. Peptide-pulsed cells (1×10^4 cells/well) or gene-transfected cells (5×10^4 cells/well) were used to stimulate CTLs at several responder/stimulator ratios in 200 μl of AIM-V/5% AS on 96 well round bottom plates (Corning Inc.). After 24 h of incubation, cell-free supernatants were harvested, and the IFN-γ production was examined by an IFN-γ ELISA kit (BD Biosciences) according to the manufacturer's instructions.

Flow cytometry

The expression of peptide-specific T cell receptors was examined on FACS-Canto II (Becton Dickinson, San Jose, CA) using PE-conjugated peptide/MHC tetramer (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions. Briefly, *in vitro* expanded CTLs were incubated with peptide/MHC tetramer at room temperature for 10 min, and then a FITC-conjugated anti-human CD8 mAb, APC-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb and 7-AAD (BD Biosciences) were added and incubated at 4°C for 20 min. HIV peptide (ILKEPVHGV)/HLA-A*02: 01 tetramer was used as a negative control.

Cytotoxicity assay

The cytotoxic activity of the induced CTL clones was tested by a 4 h ^{51}Cr release assay as described previously [32]. Data are presented as the means ± SD of triplicate samples. Student's t test was used to examine the significance of the data.

Results

CTL induction with HLA-A*02:01-binding peptides derived from HIG2

We synthesized twelve 9-mer and 10-mer peptides, corresponding to parts of the HIG2 protein that had been suggested to bind to HLA-A*02:01 by the prediction with the BIMAS program (Table 1). After *in vitro* culture to induce CTLs, IFN-γ production was observed specifically when cells were stimulated with T2 cells that had been pulsed with the HIG2-9-8 peptide (YLLGVVLTL), HIG2-9-4 peptide (VLNLYLLGV), HIG2-9-15 peptide (TLLSIFVRV) or HIG2-10-8 peptide (YLLGVVLTLL) among all of the candidate peptides shown in Table 1 (Fig. S1 showing all 12 wells of one experiment and Fig. 1a showing representative wells). After CTL-expanding culture, cells still produced IFN-γ in response to the respective peptides in a responder/stimulator ratio-dependent manner, and HIG2-9-4 peptide-specific CTLs produced a higher amount of IFN-γ than CTLs stimulated with other peptides (Figs. 1b–e). In the independent experiments using PBMCs from other 2 donors, HIG2-9-4 peptide-specific CTLs produced the highest amount of IFN-γ (data not shown). We confirmed the existence of HIG2-9-4/HLA-A*02:01-specific $CD8^+$ T cells by tetramer staining. A significant population of $CD3^+CD4^-CD8^+$ cells expressed the HIG2-9-4/HLA-A*02:01-specific T cell receptor after the expansion of cells obtained by *in vitro* CTL induction (Fig. 2).

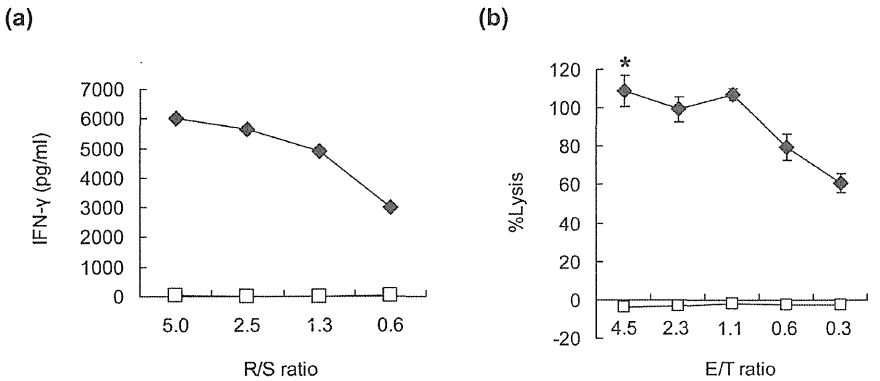

Figure 3. The IFN-γ production and cytotoxic activity of a HIG2-9-4 peptide-specific CTL clone. (a) An established CTL clone was stimulated with T2 cells pulsed with the HIG2-9-4 peptide (closed diamonds) or HIV peptide (open squares). The IFN-γ production in the culture supernatant was examined by ELISA. R/S ratio; responder/stimulator ratio. (b) The cytotoxic activity of the HIG2-9-4 peptide-specific CTL clone was examined against peptide-pulsed T2 cells (close diamond) or T2 cells pulsed with the HIV peptide (open square). E/T ratio; effector/target ratio. All experiments were performed in triplicate. The representative results from three independent experiments are shown. *$P<0.001$
doi:10.1371/journal.pone.0085267.g003

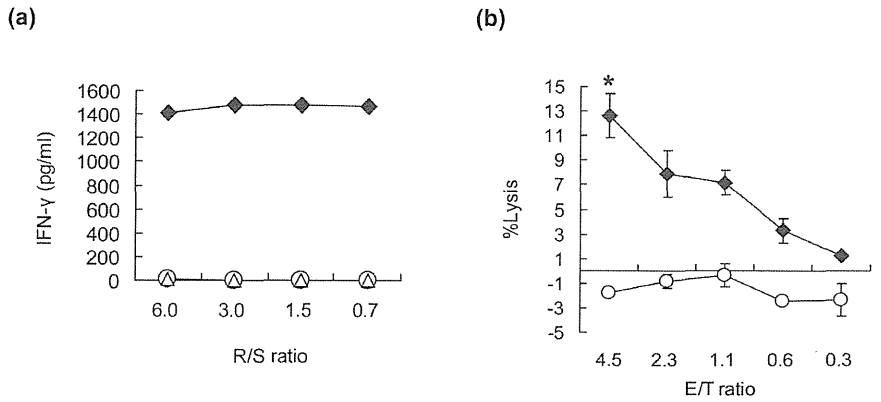

Figure 4. The recognition of HIG2 and HLA-A*02:01-expressing cells by a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:01 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:01 alone (open triangle), then the IFN-γ production was examined by ELISA. R/S ratio; responder/stimulator ratio. (b) The cytotoxic activity of the HIG2-9-4 peptide-specific CTL clone was examined against HLA-A*02:01-positive HIG2-expressing A498 cells (closed diamond) or HLA-A*02:01-negative HIG2-expressing Caki-1 cells (open circle). E/T ratio; effector/target ratio. All experiments were performed in triplicate. Representative results from three independent experiments are shown. *; $P<0.001$.
doi:10.1371/journal.pone.0085267.g004

Establishment of HIG2-9-4 peptide-specific CTL clones

We subsequently established HIG2-9-4 peptide-specific CTL clones by the limiting dilution of induced CTLs. The established HIG2-9-4 peptide-specific CTL clone produced a large amount of IFN-γ when it was stimulated with HIG2-9-4 pulsed-T2 cells, while no IFN-γ production was detected when they were stimulated with HIV-peptide-pulsed-T2 cells (Fig. 3a). Furthermore, the HIG2-9-4 peptide-specific CTL clone exerted substantial cytotoxic activity against T2 cells pulsed with the HIG2-9-4 peptide, but not those pulsed with the HIV peptide (Fig. 3b). However, we failed to establish any CTL clones that reacted with HIG2-9-8, HIG2-9-15 or HIG2-10-8 peptides, even after several attempts using multiple donors (data not shown). In addition, we found no homologous sequence to the HIG2-9-4 peptide by a homology search using the BLAST algorithm (data not shown), indicating that the HIG2-9-4 peptide is a unique epitope peptide among the candidate peptides predicted by the BIMAS program that can induce potent and stable CTLs.

Specific CTL response to HIG2 and HLA-A*02:01-expressing cells

To further verify the recognition of HIG2-expressing cells with HLA-A*02:01 by the HIG2-9-4-specific CTL clone, we prepared COS7 cells in which either or both of two plasmids designed to express the full-length of HIG2 and HLA-A*02:01 were transfected. The HIG2-9-4-specific CTL clone produced IFN-γ when the cells were exposed to the COS7 cells expressing both HIG2 and HLA-A*02:01, while no IFN-γ production was observed when they were exposed to COS7 cells expressing either HIG2 or HLA-A*02:01 (Fig. 4a). Furthermore, the HIG2-9-4 peptide-specific CTL clone demonstrated cytotoxic activity against A498 cells expressing both HLA-A*02:01 and HIG2, while no

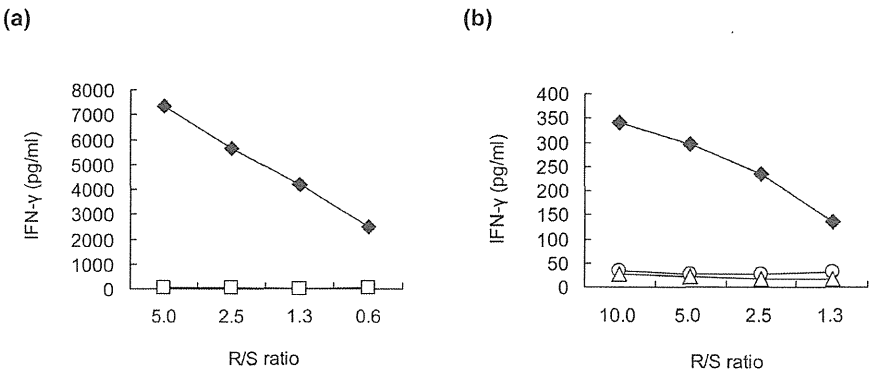

Figure 5. The HLA-A*02:06-restricted response of a HIG2-9-4 peptide-specific CTL clone. (a) A HIG2-9-4 peptide-specific CTL clone was induced from HLA-A*02:06-positive PBMCs, and stimulated with HLA-A*02:06-positive PSCCA0922 cells pulsed with the HIG2-9-4 peptide (close diamond) or HIV peptide (open square). (b) The HIG2-9-4 peptide-specific CTL clone was stimulated with COS7 cells expressing both HIG2 and HLA-A*02:06 (close diamond), or either HIG2 alone (open circle) or HLA-A*02:06 alone (open triangle). The IFN-γ production in the culture supernatant was examined by ELISA. R/S ratio; responder/stimulator ratio. The representative results from three independent experiments are shown.
doi:10.1371/journal.pone.0085267.g005

cytotoxicity was observed against HIG2-expressing Caki-1 cells without HLA-A*02:01 expression (Fig. 4b).

The HIG2-9-4 peptide cross-reacts with HLA-A*02:06

We additionally evaluated the cross-reactivity of the HIG2-9-4 peptide with HLA-A*02:06, since HLA-A*02:06 differs from HLA-A*02:01 by a single amino acid, and some reports have indicated the presentation of HLA-A*02:01-restricted peptides on HLA-A*02:06 [33,34]. Similar to the HLA-A*02:01 experiments, potent CTL clones were established from the PBMCs of HLA-A*02:06-positive donors by stimulation with the HIG2-9-4 peptide. An established CTL clone showed potent IFN-γ production when it was exposed to HIG2-9-4 peptide-pulsed HLA-A*02:06-positive PSCCA0922 cells (Fig. 5a). Furthermore, this CTL clone recognized COS7 cells that expressed both HIG2 and HLA-A*02:06 and produced IFN-γ, while no IFN-γ production was observed when stimulated with COS7 cells that expressed either HIG2 or HLA-A*02:06 (Fig. 5b). These results suggested that the HIG2-9-4 peptide is cross-reactive with HLA-A*02:06 to induce CTLs that show CTL activity against HLA-A*02:06- and HIG2-expressing cells.

Discussion

The recent FDA approvals of the cellular immunotherapy, Sipuleucel-T (Provenge), and immunomodulatory antibody, ipilimumab (Yervoy), have provided a proof of concept that the immune system can be used as a new approach to treat cancer [35,36]. Immunization with HLA-restricted epitope peptides derived from tumor antigens is a strategy that has been vigorously pursued to activate the immune system [37-40]. Unfortunately, many of the vaccine trials using epitope peptides failed to demonstrate clinical efficacy due, at least in part, to the potential immune escape mechanisms, which are attributed to the loss of tumor antigen expression by tumor cells [41-43]. Accordingly, the selection of tumor antigens which play a key role in tumor cell proliferation or survival is considered to be important to overcome immune escape. If a targeted tumor antigen is essential for tumor growth, the downregulation of this tumor antigen as a form of immune escape is expected to impair tumor progression.

Correspondingly, in the guidelines from the FDA (Guidance for Industry: Clinical Considerations for Therapeutic Cancer Vaccines), multi-antigen vaccines which contain multiple tumor antigens in order to generate multiple tumor-specific immunological responses were mentioned to effectively hinder escape mechanisms. We therefore consider that the identification of epitope peptides derived from multiple tumor antigens which are involved in tumor progression or survival can contribute to the development of multi-antigen vaccines, and can improve the efficacy of peptide vaccine therapies. We have previously identified epitope peptides derived from various tumor antigens, each of which plays a key role in tumor progression, and some of these peptides have been applied for clinical trials as multi-peptide vaccines [44–46].

In this study, we identified an HLA-A2 supertype-restricted epitope peptide derived from HIG2. HIG2 was upregulated in RCC and hardly detectable in normal organs except for the fetal kidney, and importantly, HIG2 expression was found to be directly associated with the proliferation of RCC cells [24]. Hence, RCC cells are thought to maintain HIG2 expression even under immunoselective pressure, or to otherwise exhibit tumor growth suppression resulting from the loss of HIG2 expression.

IFN-γ-producing stable CTL clones specific to the HIG2-9-4 peptide (VLNLYLLGV) were established from HLA-A2 (either A*02:01 or A*02:06)-positive PBMCs, and these clones responded specifically to COS7 cells that expressed both HIG2 and HLA-A2 (A*02:01 or A*02:06). We also revealed that HIG2-9-4-specific HLA-A*02:01-restricted CTLs exerted cytotoxic activity against RCC cells that were positive for both HIG2 and HLA-A*02:01, but not against negative cells. These results suggested that HLA-A2 (A*02:01 or A*02:06)-restricted HIG2-9-4 peptide-specific CTLs are inducible and stable, and these CTLs substantially respond to HIG2-expressing cells through the endogenous processing of the HIG2-9-4-peptide and the subsequent presentation with the HLA-A2 (A*02:01 or A*02:06) molecule on the cell surface. In addition, HIG2 is an oncofetal antigen, as described above, and no homologous sequence to the HIG2-9-4 peptide was demonstrated by a homology search using the BLAST algorithm. Thus, HIG2-9-4 peptide-specific CTLs should not induce unintended immunological responses to normal cells, such as those associated with autoimmune diseases, even if this novel and unique peptide induces strong immune responses against HIG2-expressing RCC.

HIG2 expression was found in the majority of RCC patients (86%) [25], and additionally, the HLA-A2 supertype is the most common HLA class I type in Caucasians and the second most common type in the Japanese population [26,27]. Therefore, identification of HLA-A2 supertype-restricted epitope peptides derived from HIG2 could be applicable for immunotherapies in a wide variety of RCC patients. As well as finding novel tumor antigens which are widely expressed in cancer patients, finding epitope peptides restricted to major HLA Class I types will facilitate further development of cancer immunotherapies. We are now conducting clinical trials to examine the immunogenicity and safety of a HIG2-9-4 peptide vaccine in RCC patients.

Supporting Information

Figure S1 Response to the HIG2-9-8, HIG2-9-15, HIG2-9-4 or HIG2-10-8 peptide detected by IFN-γ ELISPOT assay. The IFN-γ production from cells induced by the indicated peptide-pulsed DCs in 12 wells for each peptide was examined by an ELISPOT assay. "+" indicates the wells in which cells were stimulated with T2 cells pulsed with the indicated peptide and "−" indicates the wells in which cells were stimulated with HIV peptide-pulsed T2 cells. The wells in which the difference between peptide-pulsed cells and HIV peptide-pulsed cells were over 50 spots are indicated by squares.
(TIF)

Acknowledgments

The authors thank Dr. Kawakami (Keio University, Tokyo, Japan) for providing the expression vector, and the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University for providing the cell lines.

Author Contributions

Conceived and designed the experiments: TT RO HY. Performed the experiments: SY MH TW TH. Analyzed the data: SY MH TW TH. Wrote the paper: SY. Scientific advise: MK MM MT MI. Support to draft the manuscript: KT TK YN.

References

1. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60: 277–300.
2. Cohen HT, McGovern FJ (2005) Renal-cell carcinoma. N Engl J Med 353: 2477–2490.
3. National Comprehensive Cancer Network (2012) NCCN Clinical Practice Guidelines in Oncology. Kidney Cancer. Version2.2012. Available: http://www.tri-kobe.org/nccn/guideline/urological/english/kidney.pdf
4. Patil S, Ishill N, Deluca J, Motzer RJ (2010) Stage migration and increasing proportion of favorable-prognosis metastatic renal cell carcinoma patients: implications for clinical trial design and interpretation. Cancer 116: 347–354.
5. Rini BI, Escudier B, Tomczak P, Kaprin A, Szczylik C, et al. (2011) Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. Lancet 378: 1931–1939.
6. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, et al. (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 356: 115–124.
7. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, et al. (2009) Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. J Clin Oncol 27: 3584–3590.
8. Escudier B, Szczylik C, Hutson TE, Demkow T, Staehler M, et al. (2009) Randomized phase II trial of first-line treatment with sorafenib versus interferon Alfa-2a in patients with metastatic renal cell carcinoma. J Clin Oncol 27: 1280–1289.
9. Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, et al. (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med 356: 2271–2281.
10. Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, et al. (2007) Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet 370: 2103–2111.
11. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, et al. (2008) Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 372: 449–456.
12. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, et al. (2010) Phase 3 trial of everolimus for metastatic renal cell carcinoma: final results and analysis of prognostic factors. Cancer 116: 4256–4265.
13. Sternberg CN, Davis ID, Mardiak J, Szczylik C, Lee E, et al. (2010) Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. J Clin Oncol 28: 1061–1068.
14. McDermott DF (2009) Immunotherapy of metastatic renal cell carcinoma. Cancer 115: 2298–2305.
15. Sparano JA, Fisher RI, Sunderland M, Margolin K, Ernest ML, et al. (1993) Randomized phase III trial of treatment with high-dose interleukin-2 either alone or in combination with interferon alfa-2a in patients with advanced melanoma. J Clin Oncol 11: 1969–1977.
16. Atzpodien J, Kirchner H, Jonas U, Bergmann L, Schott H, et al. (2004) Interleukin-2- and interferon alfa-2a-based immunochemotherapy in advanced renal cell carcinoma: a Prospectively Randomized Trial of the German Cooperative Renal Carcinoma Chemoimmunotherapy Group (DGCIN). J Clin Oncol 22: 1188–1194.
17. Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR, et al. (1995) Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. J Clin Oncol 13: 688–696.
18. Bleumer I, Tiemessen DM, Oosterwijk-Wakka JC, Völler MC, De Weijer K, et al. (2007) Preliminary analysis of patients with progressive renal cell carcinoma vaccinated with CA9-peptide-pulsed mature dendritic cells. J Immunother 30: 116–122.
19. Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT (2002) Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev 188: 22–32.
20. Tsuji T, Matsuzaki J, Kelly MP, Ramakrishna V, Vitale L, et al. (2011) Antibody-targeted NY-ESO-1 to mannose receptor or DEC-205 in vitro elicits dual human CD8+ and CD4+ T cell responses with broad antigen specificity. J Immunol 186: 1218–1227.
21. Amato RJ, Shingler W, Goonewardena M, de Belin J, Naylor S, et al. (2009) Vaccination of renal cell cancer patients with modified vaccinia Ankara delivering the tumor antigen 5T4 (TroVax) alone or administered in combination with interferon-α (IFN-α): a Phase 2 trial. J Immunother 32: 765–772.
22. Denko N, Schindler C, Koong A, Laderoute K, Green C, et al. (2000) Epigenetic regulation of gene expression in cervical cancer cells by the tumor microenvironment. Clin Cancer Res 6: 480–487.
23. Gimm T, Wiese M, Teschemacher B, Deggerich A, Schödel J, et al. (2010) Hypoxia-inducible protein 2 is a novel lipid droplet protein and a specific target gene of hypoxia-inducible factor-1. FASEB J 24: 4443–4458.
24. Togashi A, Katagiri T, Ashida S, Fujioka T, Maruyama O, et al. (2005) Hypoxia-inducible protein 2 (HIG2), a novel diagnostic marker for renal cell carcinoma and potential target for molecular therapy. Cancer Res 65: 4817–4826.
25. Seo T, Konda R, Sugimura J, Iwasaki K, Nakamura Y, et al. (2010) Expression of hypoxia-inducible protein 2 in renal cell carcinoma: A promising candidate for molecular targeting therapy. Oncol Lett 1: 697–701.
26. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, et al. (2001) Analysis of the frequency of HLA-A, B and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. Hum Immunol 62: 1009–1030.
27. Itoh Y, Mizuki N, Shimada T, Azuma F, Itakura M, et al. (2005) High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. Immunogenetics 57: 717–729.
28. Tsomides TJ, Aldovini A, Johnson RP, Walker BD, Young RA, et al. (1994) Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. J Exp Med 180: 1283–1293.
29. Celis E, Tsai V, Crimi C, DeMars R, Wentworth PA, et al. (1994) Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc Natl Acad Sci U S A 91: 2105–2109.
30. Uchida N, Tsunoda T, Wada S, Furukawa Y, Nakamura Y, et al. (2004) Ring finger protein (RNF) 43 as a New Target for Cancer Immunotherapy. Clin Can Res 10: 8577–8586.
31. Suda T, Tsunoda T, Daigo Y, Nakamura Y, Tahara H (2007) Identification of human leukocyte antigen-A24-restricted epitope peptides derived from gene products upregulated in lung and esophageal cancers as novel targets for immunotherapy. Cancer Sci 98: 1803–1808.
32. Takeda K, Yamaguchi N, Akiba H, Kojima Y, Hayakawa Y, et al. (2004) Induction of tumor-specific T cell immunity by anti-DR5 antibody therapy. J Exp Med 199: 437–448.
33. Sidney J, Southwood S, Mann DL, Fernandez-Vina MA, Newman MJ, et al. (2001) Majority of peptides binding HLA-A*0201 with high affinity crossreact with other A2-supertype molecules. Hum Immunol 62: 1200–1216.
34. Fleischhauer K, Tanzarella S, Russo V, Sensi ML, van der Bruggen P, et al. (1997) Functional heterogeneity of HLA-A*02 subtypes revealed by presentation of a MAGE-3-encoded peptide to cytotoxic T cell clones. J Immunol 159: 2513–2521.
35. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, et al. (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363: 411–422.
36. Robert C, Thomas L, Bondarenko I, O'Day S, M D JW, et al. (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med 364: 2517–2526.
37. Boon T (1993) Tumor antigens recognized by cytolytic T lymphocytes: present perspectives for specific immunotherapy. Int J Cancer 54: 177–180.
38. Rimoldi D, Rubio-Godoy V, Dutoit V, Lienard D, Salvi S, et al. (2000) Efficient simultaneous presentation of NY-ESO-1/LAGE-1 primary and nonprimary open reading frame-derived CTL epitopes in melanoma. J Immunol 165: 7253–7261.
39. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Topalian SL, et al. (2003) Recombinant fowlpox viruses encoding the anchor-modified gp100 melanoma antigen can generate antitumor immune responses in patients with metastatic melanoma. Clin Cancer Res 9: 2973–2980.
40. Pecher G, Häring A, Kaiser L, Thiel E (2002) Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. Cancer Immunol Immunother 51: 669–673.
41. Rosenberg SA, Yang JC, Restifo NP (2004) Cancer immunotherapy: moving beyond current vaccines. Nat Med 10: 909–915.
42. Sampson JH, Heimberger AB, Archer GE, Aldape KD, Friedman AH, et al. (2010) Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. J Clin Oncol 28: 4722–4729.
43. DuPage M, Mazumdar C, Schmidt LM, Cheung AF, Jacks T (2012) Expression of tumour-specific antigens underlies cancer immunoediting. Nature 482: 405–409.
44. Okuno K, Sugiura F, Hida JI, Tokoro T, Ishimaru E, et al. (2011) Phase I clinical trial of a novel peptide vaccine in combination with UFT/LV for metastatic colorectal cancer. Exp Ther Med 2: 73–79.
45. Kono K, Iinuma H, Akutsu Y, Tanaka H, Hayashi N, et al. (2012) Multicenter, phase II clinical trial of cancer vaccination for advanced esophageal cancer with three peptides derived from novel cancer-testis antigens. J Transl Med 10: 141.
46. Obara W, Ohsawa R, Kanehira M, Takata R, Tsunoda T, et al. (2012) Cancer peptide vaccine therapy developed from oncoantigens identified through genome-wide expression profile analysis for bladder cancer. Jpn J Clin Oncol 42: 591–600.

J Hepatobiliary Pancreat Sci (2014) 21:193–198
DOI: 10.1002/jhbp.16

ORIGINAL ARTICLE

Isolated Roux-en-Y anastomosis of the pancreatic stump in a duct-to-mucosa fashion in patients with distal pancreatectomy with en-bloc celiac axis resection

Ken-ichi Okada · Manabu Kawai · Masaji Tani · Seiko Hirono · Motoki Miyazawa · Atsushi Shimizu · Yuji Kitahata · Hiroki Yamaue

Published online: 22 July 2013

Abstract
Background A pancreatic fistula is one of the most serious complications in distal pancreatectomy with *en bloc* celiac axis resection (DP-CAR), because the pancreatic transection is performed on the right side of the portal vein, which results in a large cross-section surface, and because post-pancreatectomy hemorrhage is hard to treat by interventional radiology. Therefore, a procedure to decrease the incidence of postoperative pancreatic fistula is urgently needed.
Methods Twenty-six consecutive patients who underwent DP-CAR between April 2008 and August 2012 were reviewed retrospectively. The first 13 consecutive patients underwent DP-CAR with no anastomosis, and the subsequent 13 consecutive patients were treated with Roux-en-Y pancreaticojejunostomy (PJ) in a duct-to-mucosa fashion.
Results Extremely high amylase levels (>4000 IU/l) of all drainage fluid specimens on postoperative day (POD) 1, 3 and 4 were detected more frequently in cases with no anastomosis ($n = 7$) compared to those with PJ ($n = 1$) ($P = 0.056$).
Conclusion The incidence of grade B/C pancreatic fistulas was 15.4% in cases with isolated Roux-en-Y anastomosis of the pancreatic stump performed in a duct-to-mucosa fashion, and we are currently examining whether this anastomosis method reduces the pancreatic fistula rate in a multicenter, randomized controlled trial for distal pancreatectomy patients (ClinicalTrials.gov NCT01384617).

K. Okada · M. Kawai · M. Tani · S. Hirono · M. Miyazawa · A. Shimizu · Y. Kitahata · H. Yamaue (✉)
Second Department of Surgery, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8510, Japan
e-mail: yamaue-h@wakayama-med.ac.jp

Keywords Distal pancreatectomy · Duct-to-mucosa anastomosis · Pancreatic fistula · Pancreaticojejunostomy

Introduction

The overall incidence of pancreatic fistula [1, 2] in patients undergoing distal pancreatectomy is 10% to 30% in the literature [3–5]. Recently, distal pancreatectomy with *en bloc* celiac axis resection (DP-CAR) has been performed to improve survival by achieving an R0 resection in patients with advanced pancreatic body/tail carcinoma [6]. Pancreatic fistulas sometimes cause an intra-abdominal hemorrhage [7], and can become a directly fatal complication, because transarterial embolization via pancreaticoduodenal arcade is difficult to perform by interventional radiology (IVR) in patients who have undergone DP-CAR. Therefore, a procedure that decreases the incidence of postoperative pancreatic fistulas is urgently needed for distal pancreatectomy including DP-CAR. Moreover, the tumors indicated for DP-CAR often require the pancreatic transection to be performed on the right side of the portal vein. However, it is technically difficult to transect the pancreas using a stapler device on the right side of the portal vein, further complicating the procedure and increasing the risk.

A large number of investigations have been performed to avoid fistula formation, such as hand-sewn suturing of the cut end, stapler closure [3, 5, 8, 9], pancreaticoenteric anastomosis [3, 10], seromuscular patches [11–13], fibrin glue sealing [14, 15] and mesh reinforcement [16]. In a randomized controlled trial (DISPACT), the stapler closure after distal pancreatectomy did not decrease the incidence of pancreatic fistula compared to hand-sewn suturing [5]. The appropriate management of the pancreatic stump

following distal pancreatectomy remains controversial. Among the various studies of the management of the pancreatic stump, the most attractive results with a low incidence of pancreatic fistula were reported for pancreaticoenteric anastomosis [3, 10]. We hypothesized that pancreaticojejunostomy (PJ) performed in a duct-to-mucosa fashion would provide a favorable outcome by decreasing the back pressure not only in the main pancreatic duct, but also in the branch ducts. We selected the jejunum to anastomose to the pancreatic stump because it is easy to manipulate.

In this study, 13 consecutive patients with pancreatic body/tail carcinoma prospectively underwent DP-CAR with PJ. The collected data were compared to those of the previous 13 consecutive patients who underwent DP-CAR with no anastomosis.

Patients and methods

Patients

We prospectively assigned the 26 consecutive patients between April 2008 and August 2012 into two groups based on the time period of the treatment to assess the effect of pancreaticojejunostomy (PJ) at the pancreatic stump; 13 patients with no anastomosis (until February 2011) and 13 patients with Roux-en-Y PJ performed in a duct-to-mucosa fashion (after February 2011) who expected to undergo DP-CAR for pancreatic body/tail carcinoma at Wakayama Medical University Hospital (WMUH) were included. During the period between May 2010 and December 2011, the patients with borderline resectable [17] pancreatic body/tail carcinoma were planned to receive S-1 standard-dose chemotherapy for 9 weeks and multi-field radiotherapy focused on retropancreatic tissue for a total of 50 Gy over a 5-week period. After 3 weeks of rest for both therapies, the patients without progression of disease and new distant metastasis underwent DP-CAR. We reviewed the postoperative clinical data registered prospectively for 26 patients who underwent DP-CAR with no anastomosis ($n = 13$) and who underwent Roux-en-Y PJ in a duct-to-mucosa fashion ($n = 13$).

Surgical procedures and postoperative management

The procedure used for DP-CAR was similar in the 26 patients, and was reported previously [6]. In the no anastomosis group, the pancreatic parenchyma was resected by bipolar scissors (Ethicon Endo-Surgery, Cincinnati, OH, USA) ($n = 6$), an ultrasonic dissector (HARMONIC FOCUS (r) (Ethicon Endo-Surgery, LLC, Guaynabo, Puerto Rico)) with main pancreatic duct ligation ($n = 1$), or with a stapler device (Echelon 60 with a gold cartridge (compressible thickness to 1.8 mm; Ethicon Endo-Surgery) ($n = 6$), according to the status of the pancreatic transection site.

In the PJ group, the pancreatic parenchyma was resected using an ultrasonic dissector ($n = 13$) and the main pancreatic duct was resected using a scalpel to avoid the sealing of the main pancreatic duct. After achievement of distal pancreatectomy with *en bloc* celiac axis resection, a pancreaticojejunostomy end-to-side anastomosis by a Roux-en-Y limb was carried out in a retrocolic route with a length of at least 30 cm [10, 18]. The anastomosis was performed in a non-stented duct-to-mucosa fashion using a single layer of interrupted 5-0 PDS stitches (Ethicon Endo-Surgery). A seromuscular-parenchymal anastomosis was placed in one or two [18] layers according to the thickness of the pancreas on both sides of the transaction line to the jejunum to cover the cut end of the pancreas using interrupted 4-0 VASCUFIL stitches (Covidien, Mansfield, MA, USA). A tube stent was not inserted for the duct-to-mucosa anastomosis to avoid having it migrate into the duodenal side. A drain (BLAKE Silicone Drains 24Fr) was placed via the pancreatic stump terminating in the left infraphrenic space. No patients received prophylactic subcutaneous octreotide. All patients received postoperative epidural anesthesia.

Data collection

The peritoneal drainage volume was registered daily, and the amylase level in the drainage fluid was measured and recorded on postoperative day (POD) 1, 3, and 4. The drain tube was usually removed on POD 4. The patient characteristics, duration of hospital stay, incidence of pancreatic fistula and perioperative morbidity were recorded prospectively. A pancreatic fistula was defined by the International Study Group of Pancreatic Fistula (ISGPF) guidelines as: any measurable output on or after POD 3 from an operatively positioned drain displaying pancreatic amylase more than three times the upper serum reference value, and was graded according to the previously proposed definition [1, 2]. Delayed gastric emptying (DGE) was defined according to a consensus definition and clinical grading of postoperative DGE proposed by the International Study Group of Pancreatic Surgery (ISGPS) [19]. The patients were discharged only when they fulfilled the criteria as follows: were able to return to preoperative activities of daily living, had no deep-site infections, normal laboratory data, no drains and were able to take in oral nutrition above the basal metabolic requirement.

Statistical analysis

The data are expressed as medians. Statistical comparisons between two groups were made using the χ^2 statistic, Fisher's exact test or the Mann–Whitney *U*-test, as appropriate. A value of $P < 0.05$ was considered to indicate statistical significance. All the analyses were performed using the statistical software package, SPSS II (version 20.0; SPSS, Chicago, IL, USA).

Results

Patient characteristics and surgical outcomes

Table 1 shows the characteristics of the 26 consecutive patients with pancreatic body/tail cancer. These patients included 18 cases of invasive ductal carcinoma, five of invasive ductal carcinoma derived from intraductal papillary mucinous neoplasm, one acinar cell carcinoma, one anaplastic carcinoma, and one mucinous carcinoma. Combined resections of the portal vein were performed in four patients. The remnant pancreatic parenchyma was soft in 20 patients (76.9%). The median length of the operation for DP-CAR with PJ was 382 min (range, 267–513 min) compared to 366 min (range, 136–846 min) for DP-CAR with no anastomosis ($P = 0.840$). In the PJ group, a seromuscular-parenchymal anastomosis was placed in one layer in eight patients, and two layers in five patients, according to the thickness of the pancreas.

Postoperative complications

Table 2 shows the surgical results and postoperative complications recorded in this study. A clinically significant pancreatic fistula (ISGPF classification Grade B/C) occurred in two patients (15.4%) with PJ and five patients (38.5%) with no anastomosis ($P = 0.189$). There were no significant differences in the incidence of each complication (Table 2). A death associated with surgery was present in each group; an acute myocardial infarction occurred 10 days postoperatively in one patient in the PJ group, and intra-abdominal hemorrhage occurred 28 days postoperatively in

Table 1 Patient characteristics and outcomes of surgery

Parameters	No anastomosis ($n = 13$)	With pancreaticojejunostomy ($n = 13$)	*P*-value
Age at surgery	63	68	0.695
Gender			
Male	10	7	0.411
Female	3	6	
NACRT	4	10	0.047[a]
Portal vein resection	1	3	0.593
Histopathology			
IDC	6	12	
Invasive ductal carcinoma derived from IPMN	5	0	
Acinar cell carcinoma	1	1	
Anaplastic carcinoma	0	0	
Mucinous carcinoma	1	0	
Hardness of the pancreas			
Soft	9	11	0.645
Hard	4	2	
Transect position			
Midline of the portal vein	6	7	0.999
Right side of the portal vein	7	6	
Thickness of the pancreas at the transection position (mm)	12.3 ± 1.7	10.9 ± 2.7	0.113
Length of operation (min)	366 (136–846)	382 (267–513)	0.840
EBL (ml)	1,860 (280–9,900)	460 (30–1,250)	0.006[a]
Hospital days after surgery	24 (10–51)	21 (11–192)	0.724

EBL estimated blood loss, *IDC* invasive ductal carcinoma of the pancreas, *IPMN* intraductal papillary-mucinous neoplasm, *n* number of cases, *NACRT* neoadjuvant chemoradiation therapy

All results are shown as medians (range)

[a] $P < 0.05$

Table 2 Postoperative complications and postoperative outcomes

Postoperative complications	No anastomosis ($n = 13$)	With pancreaticojejunostomy ($n = 13$)	P-value
Pancreatic fistula[a]			
Grade A	0	1 (7.7%)	0.999
Grade B	4 (30.8%)	1 (7.7%)	0.322
Grade C	1 (7.7%)	1 (7.7%)	0.999
Grade B/C	5 (38.5%)	2 (15.4%)	0.185
Delayed gastric emptying[b]			
Grade A	3 (23.1%)	1 (7.7%)	0.593
Grade B	0	1 (7.7%)	0.999
Grade C	1 (7.7%)	1 (7.7%)	0.999
Necrosis of the gallbladder	0	1 (7.7%)	0.999
Gastroduodenal perforation	2 (15.4%)	0	0.480
Surgical site infection			
Wound infection	1 (7.7%)	0	0.999
Intra-abdominal abscess	3 (23.1%)	1 (7.7%)	0.593
Intra-abdominal hemorrhage[c]			
Grade A	0	0	
Grade B	0	0	
Grade C	3 (23.1%)	3 (23.1%)	0.999
Pulmonary complications	0	0	
Cardiac complications[d]	0	1 (7.7%)	0.999
Percutaneous drainage	2 (15.4%)	3 (23.1%)	0.999
Reoperation	3 (23.1%)	1 (7.7%)	0.593
Mortality	1 (7.7%)	1 (7.7%)	0.999

n number of cases

[a,b,c] Pancreatic fistula, delayed gastric emptying, and intra-abdominal hemorrhage were defined according to the International Study Group of Pancreatic Surgeons

[d] A patient died from an acute myocardial infarction

one patient in the no anastomosis group. An ischemic cholecystitis presumably from temporary decreased flow of the proper hepatic artery occurred 7 days postoperatively in one patient after DP-CAR. This complication was treated successfully by cholecystectomy. The patients combined with grade C pancreatic fistula after this operation and it takes a longer period (192 days) to heal (Table 1). Otherwise, there were no significant differences in regard to the mortality and morbidity rates between the groups.

The amylase level in the drainage fluid

Extremely high amylase levels (>4000 IU/l) of all drainage fluid specimens on POD 1, 3 and 4 were detected more frequently in cases with no anastomosis ($n = 7$, 17.9%) compared to those with PJ ($n = 1$, 2.6%) ($P = 0.056$) (Fig. 1). The median amylase level in the drainage fluid of patients with PJ/no anastomosis were 315/589 (IU/l) on POD 1, 121/286 (IU/l) on POD 3 and 52/247 on POD 4, respectively.

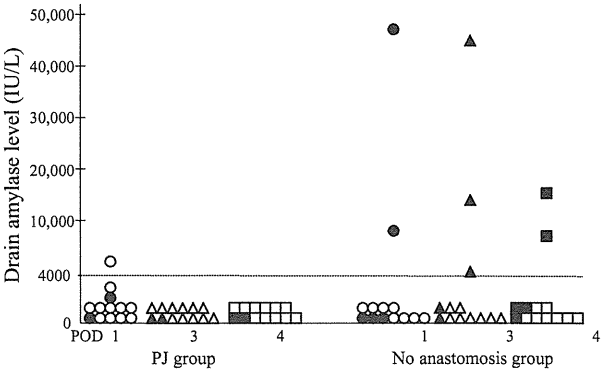

Fig. 1 Extremely high amylase levels (>4000 IU/l) of all drainage fluid specimens on postoperative day (POD) 1, 3 and 4 were detected more frequently in cases with no anastomosis ($n = 7$, 17.9%) compared to those with pancreaticojejunostomy ($n = 1$, 2.6%) ($P = 0.056$). The black dots represent the amylase levels (IU/l) of patients with grade B/C pancreatic fistulas, and white dots represent those of patients with no pancreatic fistula or a grade A pancreatic fistula (circular dot: postoperative day 1, triangular dot: postoperative day 3, tetragonal dot: postoperative day 4). PJ: pancreaticojejunostomy

Discussion

The presence of postoperative hemorrhage from the resected stump of the common hepatic artery due to a pancreatic fistula after DP-CAR is difficult to rescue by IVR techniques because of the resection of the common hepatic artery. Therefore, a novel procedure to reduce the risk of pancreatic fistula formation is urgently needed for DP-CAR, in which the pancreatic transection with a large cross-section surface is usually located on the right side of the portal vein.

The most appropriate treatment of the stump closure following DP remains controversial. A recent randomized controlled trial revealed that stapler closure did not reduce the rate of pancreatic fistula formation compared to hand-sewn closure for standard distal pancreatectomy. Two reasons for the formation of a pancreatic fistula after DP are the development of increased back pressure in the main pancreatic duct [20] and the autolysis of the pancreatic stump [11–13]. Some investigators have reported favorable outcomes with regard to pancreatic fistula by pancreaticoenterostomy [3, 10]. It has been reported that covering the stapled pancreatic remnants with a seromuscular patch can decrease the overall pancreas-related complications, such as fistula formation [11]. On the other hand, prophylactic transpapillary pancreatic stenting did not reduce clinically significant pancreatic fistula formation in a recent randomized controlled trial of distal pancreatectomy [21]. Therefore, in this study, we performed the pancreaticoenterostomy and included a duct-to-mucosa anastomosis to decrease the incidence of pancreatic fistula by decreasing the back pressure of the pancreatic duct and creating a tight seal around the pancreatic stump.

Several studies have reported that a high amylase level in drainage fluid was a predictive risk factor for a pancreatic fistula [22]. In this study, extremely high amylase levels (>4000 IU/l) [22] of all drainage fluid specimens were detected more frequently in cases with no anastomosis compared to those with PJ. The degree of autolysis for the tissues, such as arteries around the pancreatic stump, would be correlated to the amylase level. Therefore, the lower amylase levels of the PJ group in this study suggested that the procedure may have decreased the incidence of clinically significant pancreatic fistulas after DP-CAR. A randomized controlled trial should be performed for patients with distal pancreatectomy to determine the optimal procedure for the pancreatic stump.

We should take an extra caution with the complication of the intestinal side compared to simple closure of the pancreatic stump. However, there were no differences of clinical course in patients with Grade C pancreatic fistula compared to simple closure of pancreatic stump. The patients were able to continue oral intake even in the period with clinically relevant pancreatic fistula because of isolated Roux-en Y anastomosis. With regard to other specific complications, the additional procedure, including jejuno-jejunostomy or closure of the mesentery, did not cause paralytic ileus or small bowel obstruction in the early/later postoperative period so far in this series. A longer-term follow-up will be needed to confirm that this procedure does not result in additional complications.

In conclusion, the greatest advantage of isolated Roux-en-Y anastomosis of the pancreatic stump in a duct-to-mucosa fashion is to suppress the leakage with extremely high amylase level, which could reduce the incidence of clinically significant pancreatic fistula after DP-CAR. However, the groups are not comparable in the use of neoadjuvant chemoradiation and the way of pancreatic transection. Therefore, a valid conclusion could be obtained if we experienced a greater number of patients. We are proceeding to examine whether this anastomosis method reduces the pancreatic fistula formation in a multicenter, randomized controlled trial for distal pancreatectomy patients (ClinicalTrials.gov NCT01384617).

Conflict of interest None declared.

References

1. Bassi C, Dervenis C, Butturini G, Fingerhut A, Yeo C, Izbicki J, et al.; International Study Group on Pancreatic Fistula Definition. Postoperative pancreatic fistula: an international study group (ISGPF) definition. Surgery. 2005;138:8–13.
2. Reid-Lombardo KM, Farnell MB, Crippa S, Barnett M, Maupin G, Bassi C, et al.; Pancreatic Anastomotic Leak Study Group. Pancreatic anastomotic leakage after pancreaticoduodenectomy in 1,507 patients: a report from the Pancreatic Anastomotic Leak Study Group. J Gastrointest Surg. 2007;11:1451–8.
3. Kleeff J, Diener MK, Z'graggen K, Hinz U, Wagner M, Bachmann J, et al. Distal pancreatectomy: risk factors for surgical failure in 302 consecutive cases. Ann Surg. 2007;245:573–82.
4. Knaebel HP, Diener MK, Wente MN, Büchler MW, Seiler CM. Systematic review and meta-analysis of technique for closure of the pancreatic remnant after distal pancreatectomy. Br J Surg. 2005;92:539–46.
5. Diener MK, Seiler CM, Rossion I, Kleeff J, Glanemann M, Butturini G, et al. Efficacy of stapler versus hand-sewn closure after distal pancreatectomy (DISPACT): a randomised, controlled multicentre trial. Lancet. 2011;377:1514–22.
6. Okada K, Kawai M, Tani M, Hirono S, Miyazawa M, Shimizu A, et al. Surgical strategy for patients with pancreatic body/tail carcinoma: who should undergo distal pancreatectomy with en-bloc celiac axis resection? Surgery. 2013;153:365–72.
7. Wente MN, Veit JA, Bassi C, Dervenis C, Fingerhut A, Gouma DJ, et al. Postpancreatectomy hemorrhage (PPH): an International Study Group of Pancreatic Surgery (ISGPS) definition. Surgery. 2007;142:20–5.
8. Zhou W, Lv R, Wang X, Mou Y, Cai X, Herr I. Stapler vs suture closure of pancreatic remnant after distal pancreatectomy: a meta-analysis. Am J Surg. 2010;200:529–36.

9. Pannegeon V, Pessaux P, Sauvanet A, Vullierme MP, Kianmanesh R, Belghiti J. Pancreatic fistula after distal pancreatectomy: predictive risk factors and value of conservative treatment. Arch Surg. 2006;141:1071–6.
10. Wagner M, Gloor B, Ambühl M, Worni M, Lutz JA, Angst E, et al. Roux-en-Y drainage of the pancreatic stump decreases pancreatic fistula after distal pancreatic resection. J Gastrointest Surg. 2007;11:303–8.
11. Oláh A, Issekutz A, Belágyi T, Hajdú N, Romics L Jr. Randomized clinical trial of techniques for closure of the pancreatic remnant following distal pancreatectomy. Br J Surg. 2009;96: 602–7.
12. Moriura S, Kimura A, Ikeda S, Iwatsuka Y, Ikezawa T, Naiki K. Closure of the distal pancreatic stump with a seromuscular flap. Surg Today. 1995;25:992–4.
13. Kluger Y, Alfici R, Abbley B, Soffer D, Aladgem D. Gastric serosal patch in distal pancreatectomy for injury: a neglected technique. Injury. 1997;28:127–9.
14. Ohwada S, Ogawa T, Tanahashi Y, Nakamura S, Takeyoshi I, Ohya T, et al. Fibrin glue sandwich prevents pancreatic fistula following distal pancreatectomy. World J Surg. 1998;22:494–8.
15. Velanovich V. The use of tissue sealant to prevent fistula formation after laparoscopic distal pancreatectomy. Surg Endosc. 2007;21:1222.
16. Hamilton NA, Porembka MR, Johnston FM, Gao F, Strasberg SM, Linehan DC, et al. Mesh reinforcement of pancreatic transection decreases incidence of pancreatic occlusion failure for left pancreatectomy: a single-blinded, randomized controlled trial. Ann Surg. 2012;255:1037–42.
17. Tempero MA, Arnoletti JP, Behrman S, Ben-Josef E, Benson AB 3rd, Berlin JD, et al. Pancreatic adenocarcinoma. J Natl Compr Canc Netw. 2010;8:972–1017.
18. Büchler MW, Friess H, Wagner M, Kulli C, Wagener V, Z'Graggen K. Pancreatic fistula after pancreatic head resection. Br J Surg. 2000;87:883–9.
19. Wente MN, Bassi C, Dervenis C, Fingerhut A, Gouma DJ, Izbicki JR, et al. Delayed gastric emptying (DGE) after pancreatic surgery: a suggested definition by the International Study Group of Pancreatic Surgery (ISGPS). Surgery. 2007;142:761–8.
20. Hashimoto Y, Traverso LW. After distal pancreatectomy pancreatic leakage from the stump of the pancreas may be due to drain failure or pancreatic ductal back pressure. J Gastrointest Surg. 2012;16:993–1003.
21. Frozanpor F, Lundell L, Segersvärd R, Arnelo U. The effect of prophylactic transpapillary pancreatic stent insertion on clinically significant leak rate following distal pancreatectomy: results of a prospective controlled clinical trial. Ann Surg. 2012;255: 1032–6.
22. Kawai M, Kondo S, Yamaue H, Wada K, Sano K, Motoi F, et al. Predictive risk factors for clinically relevant pancreatic fistula analyzed in 1,239 patients with pancreaticoduodenectomy: multicenter data collection as a project study of pancreatic surgery by the Japanese Society of Hepato-Biliary-Pancreatic Surgery. J Hepatobiliary Pancreat Sci. 2011;18:601–8.