

Transition of serum cytokines following pancreaticoduodenectomy: A subsidiary study of JAPAN-PD

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Abstract. Our previous study aimed to examine the effect of TJ-100, a widely used herbal medicine, on intestinal function following pancreaticoduodenectomy (PD) in a multicenter, randomized, double-blinded, placebo-controlled manner (JAPAN-PD study). This concomitant study investigated the effect of TJ-100 on serum cytokine levels in patients who underwent PD. Due to the fact that several clinical variables can affect the absolute values of baseline serum cytokine levels, the ratios of the cytokine levels on postoperative day (POD)3 to those on POD1 were also used for analysis. The present study enrolled 180/224 randomized patients, of whom 91 received TJ-100 and 89 received placebo. As the main findings of the analysis, Wilcoxon signed-rank test revealed no significant difference in the levels of serum cytokines between the groups; however, patients in the TJ-100 group without severe inflammatory complications exhibited significantly higher ratios of interleukin (IL)-4 (n=123), IL-9 (n=72), IL-10 (n=97), PDGF-BB (n=143)

and tumor necrosis factor- α (n=135), compared with patients in the Placebo Group ($P<0.05$). According to the results of the present study, TJ-100 has an effect on the change in serum cytokine levels from POD1 to POD3 following PD. However, the role of different transition pattern of cytokines in postoperative recovery following PD has to be investigated by further mechanical studies focusing on these extracted cytokines (ClinicalTrials.gov; no. NCT01607307; May 30, 2012).

Introduction

TJ-100 (Daikenchuto, or TU-100), which comprises three different medicinal herbs, is widely used in clinical practice to treat 'abdominal bloating and cold sensation in the abdomen' (1,2). Clinical studies support the use of TJ-100 in the perioperative period to accelerate the recovery of gastrointestinal function (3-6). The beneficial effect of TJ-100 is mediated by enteric/sensory nerve stimulation via acetylcholine receptors and/or serotonin receptors (7,8) and by the release of adrenomedullin from epithelial cells (9-11). Moreover, TJ-100 decrease the level of postoperative inflammatory cytokines, ameliorating inflammation and postoperative ileus in animal model (12).

JAPAN-PD study, a multicenter, randomized, double-blinded, and placebo-control trial involving nearly 300 patients, was conducted to study the effect of TJ-100 on recovery of gastrointestinal function after pancreaticoduodenectomy (PD) (13). In contrast to the hypothesis, perioperative administration of TJ-100 provided no positive effect on time to first postoperative flatus and occurrence rate of paralytic ileus. Concurrently, the cytokine levels in 180 of the trial patient group were investigated, measuring the levels in serum and drainage

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fluid on postoperative day (POD)1 and POD3. Consistently, we found the lack of significant difference in 27 cytokine levels for both serum and drainage fluid between the TJ-100- and placebo-treated patients ((n=180, $P>0.05$)) (13).

However, we noted that the cytokine levels differed substantially among individuals. The range of factors potentially influencing cytokine levels, including gene polymorphisms (14,15), malignancy itself (16), and different degree of pre/postoperative inflammation, might have masked the effect of TJ-100. We also considered that cytokine levels of drainage fluid are not suitable for evaluating the effects of TJ-100, because they would be more influenced by the local environment near the tip of the tube than by the whole environment or systemic response. Thus, we determined to re-analyze the effect of TJ-100 using the data from the previous study, but with a special focus on the serum levels and efforts to minimize the influence of other potentially confounding factors.

Materials and methods

Trial design and patient recruitment. The details of the protocol and patient recruitment were described previously (13). Patients aged 20 years or older, who were undergoing PD for perianal tumor or pancreas head tumors, were recruited. The patients were randomized according to four clinical variables: i) type of primary tumor; ii) presence of neoadjuvant chemotherapy; iii) preservation of gastric pylorus; and iv) presence of enteral alimentation via a feeding tube. Administration of TJ-100 (15 g/day divided into three doses; before every meal or every 8 h) or placebo was initiated three days before surgery, and continued until POD14. Patient serum was collected on POD1 and POD3, and assayed for 27 different cytokines by LSI Medience Corporation (Tokyo, Japan) using the Human Cytokine 27-plex assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Bio-Plex 200 System (Bio-Rad Laboratories, Inc.).

The study protocol was registered at UMIN Clinical Trial Registry (no. 000007975) and at ClinicalTrials.gov (no. NCT01607307). The institutional review board of each participating institute approved the trial protocol, and the randomization and intervention was started after obtaining the patients' written informed consent. Additional written informed consent was obtained for the present study.

Statistical analysis. The characteristic features were compared between the patients receiving TJ-100 (TJ-100 Group) and placebo (Placebo Group) using Student's t-test or Fisher's exact tests. First, we compared the levels of serum cytokines on POD1 and POD3 between the groups by using the Wilcoxon signed-rank test. Second, we compared ratios of the cytokine levels on POD3 to those on POD1 for further analysis using Wilcoxon signed-rank test. Note that the values under the measurement threshold were excluded from the analysis, because the assignment of lower limit may significantly distort the ratios. P-values of <0.05 was considered significant. The statistical analysis was performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

Results

Clinical variables. We analyzed clinical data of the patients who participated in this additional explorative study after

dividing them into the TJ-100 Group and Placebo Group (Fig. 1 and Table I). As in the original JAPAN-PD study (13), the TJ-100 Group included more patients of an advanced age than did the Placebo Group (69.4 ± 8.0 years vs. 65.1 ± 11.4 , $P=0.039$). We consider this difference was coincidentally formed because the age was not used as a balancing variable of randomization. Other clinical factors that could potentially alter the cytokine levels did not differ between the groups.

Absolute values of serum cytokines. The values under the measurement threshold were excluded in this analysis. As a result, IL-2 and IL-15 were analyzed for only a small number of the patients, and thus statistical comparison was invalid. As previously reported (13), Wilcoxon signed-rank test revealed no significant difference in cytokine levels between the groups for POD1 (Table II) or POD3.

Of note, the cytokine levels ranged widely among individuals. In TNF- α on POD1, for instance, 1,072 pg/ml was observed as a maximum value, while the lower level was below the lower measurement limit (20.64 pg/ml). Similarly, the ranges of other cytokines levels were found quite large after PD.

Changes in serum cytokine levels after treatment

Whole groups. The change in serum cytokine level was defined by the ratio of cytokine levels on POD3 divided by those on POD1 in each individual, and then the ratios were compared between the groups. The ratios over time of IL-4, IL-9, and IL-17 were higher in the TJ-100 Group compared to the Placebo Group, suggesting that these cytokines increased more from POD1 to POD3 following treatment with TJ-100 than with placebo.

Patients without Grade B/C pancreatic fistula or abdominal abscess. The same analysis was performed for the patients without complications related to strong abdominal inflammation, namely Grade B/C pancreatic fistula or abdominal abscess. In this subgroup, the cytokine level ratios were significantly higher for IL-4, IL-9, IL-10, PDGF-BB, and TNF- α in the TJ-100 Group than the Placebo Group (Table III). For IL-4, the median ratio was 1.7 in TJ-100 Group, compared to 1.1 with placebo, suggesting that serum level of IL-4 more increased from POD1 to POD3 following TJ-100 treatment (Fig. 2A). For IL-9, IL-10, and PDGF-BB, the median ratios slightly exceeded 1.0 in the TJ-100 Group, while the median ratios of these cytokines were below 1.0 in the Placebo Group (Fig. 2B-D). For TNF- α , a statistical difference was demonstrated, although the difference in median ratio was quite small between the groups (TJ-100 Group vs. Placebo Group: 1.2 vs. 1.0, $P=0.041$; Fig. 2E).

Patients with Grade B/C pancreatic fistula or abdominal abscess. There was no significant difference in transition of the serum cytokine level when patients with Grade B/C pancreatic fistula or abdominal abscess were analyzed.

The effect of patient age on ratios of cytokine levels. We also assessed the impact of patient age on the cytokine levels and the ratios. Patients were divided into two groups, younger than 70 or 70 years and older. Comparison of the ratios for IL-4, IL-9, IL-10, PDGF-BB, and TNF- α showed no significant difference

Table I. Comparison between clinical variables and the TJ-100 and Placebo Groups.

Patient characteristic	TJ-100, n=91	Placebo, n=89	P-value
Age, mean \pm SD (years)	69.4 \pm 8.0	65.1 \pm 11.4	0.004
Sex, male/female (%)	50/41 (55/45)	59/30 (66/34)	0.130
BMI, mean \pm SD (kg/m ²)	22.1 \pm 3.0	22.3 \pm 3.5	0.632
Hx of abdominal surgery (%)	41 (45)	30 (34)	0.130
Tumor characteristics			
Pancreatic Ca, yes (%)	52 (57)	51 (57)	1.000
Preoperative treatment, yes (%)	74 (81)	71 (80)	0.852
Tumor stage, 0/I/II/III/Iva/IVb/ND (%)	3/12/7/25/27/3/13 (3/13/8/28/30/3/14)	3/9/6/25/24/4/18 (3/10/7/28/27/20)	0.303
Surgical variable			
Surgical time, mean \pm SD (min)	416.1 \pm 102.5	405.2 \pm 112.6	0.496
Bleeding, mean \pm SD (ml)	719.1 \pm 617.9	696.1 \pm 694.6	0.815
Use of epidural anesthesia (%)	61 (67)	65 (73)	0.418
Lymph node dissection, D0/D1/D2/D3 (%)	1/12/78/0 (1/13/86/0)	2/8/78/1 (2/9/88/1)	0.617
Dissection of nerve plexus around the SMA (%)	51 (56.0)	59 (66.3)	0.172

SD, standard deviation; BMI, body mass index; SMA, superior mesenteric plexus.

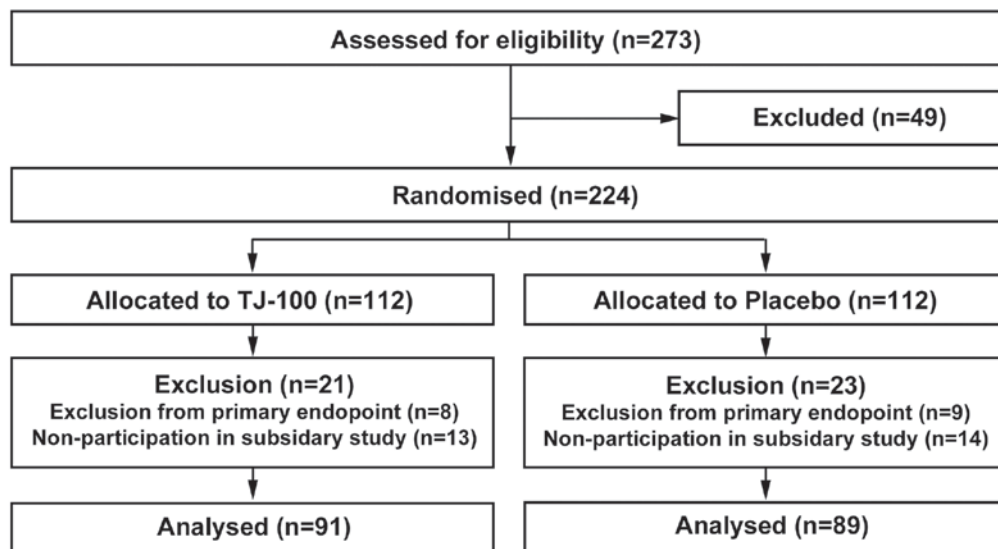


Figure 1. Flow diagram of the study. Among 273 patients, 49 were excluded from randomization due to exclusion criteria (n=41), ineligibility (n=4) and declination (n=4). Following randomization, a total of 17 patients were excluded from the primary analysis, as 13 patients did not undergo pancreatoduodenectomy, three did not receive protocol treatment and one patient withdrew consent.

between younger and elder patients (Wilcoxon signed-rank test, $P=0.75, 0.465, 0.695, 0.297$, and 0.688 , respectively).

Discussion

There are two intriguing findings in the initial analysis of this study. First, comparing the serum cytokine levels by Wilcoxon signed-rank testing revealed no significant difference at POD1 or POD3 between the patients receiving TJ-100 treatment and those receiving placebo. Second, the cytokine levels

significantly varied among the individuals even though the patients underwent similar, invasive surgical procedures. The increase in cytokine levels following surgery was quite small in some patients, even with severe complications, while other patients had substantially increased levels of the cytokines despite an uneventful postoperative recovery (data not shown).

Preoperative tumor burden and inflammation might explain these observations in part, because malignancies have been shown to alter the levels of cytokines compared to benign diseases needing surgical interventions (16). Namely,

Table II. Comparison of serum cytokine levels on POD1 between the TJ-100 and Placebo Groups.

Factor	TJ-100 (n=91)			Placebo (n=89)			P-value
	n	Median	(Min-Max)	n	Median	(Min-Max)	
Interleukin							
IL-2	5	45.5	(35.7-739.2)	13	37.2	(17.9-1250.9)	0.200
IL-7	90	14.7	(2.6-129.7)	89	18	(3.1-248.3)	0.316
IL-9	54	22.6	(10.3-4061.4)	52	25.4	(11-2143.1)	0.183
IL-13	91	7.5	(1-124.4)	88	7.6	(1.6-152)	0.466
IL-15	2	99.3	(34.1-164.5)	4	166.8	(74.6-595.7)	0.487
IL-5	91	5.9	(0.5-108.1)	89	6.6	(0.5-230.1)	0.331
GM-CSF	52	34.1	(13.8-146.8)	45	51.5	(14.4-173.9)	0.065
IL-1β	78	5.7	(2.2-53.3)	77	6.5	(2.3-51.2)	0.284
IL-6	91	216.1	(66.4-2024.7)	89	208.4	(20.2-3084.5)	0.773
IL-12	67	38.6	(11.2-635.8)	63	44.8	(11.2-747.2)	0.278
IL-17	56	102.8	(34.3-1384.4)	54	169.9	(33-602.9)	0.354
IL-1Ra	87	339.2	(36.4-2706.8)	85	319.9	(56.5-11374.9)	0.547
IL-4	82	4.1	(1.2-46.7)	77	4.9	(1.2-20)	0.152
IL-10	80	34.5	(8.7-271.4)	74	41.2	(8.7-1152.8)	0.121
Chemokine							
IL-8	91	55.2	(22.4-422.7)	89	56.5	(20-188.1)	0.925
IP-10	91	327.2	(69.3-2574.7)	89	306	(54.3-2452.9)	0.857
MCP-1	91	91.9	(28.7-453.5)	89	96	(34.8-1877.4)	0.854
MIP-1α	90	8.6	(2.5-34.9)	89	8.7	(2-31.3)	0.640
MIP-1β	91	114.8	(36.7-302)	89	100.5	(27-287.4)	0.381
RANTES	91	9,121.7	(740.1-154119.1)	89	8,984.5	(1263.2-212597.8)	0.907
Eotaxin	44	56.6	(32.1-257.8)	47	56.5	(28.2-1500.3)	0.821
Growth factor							
Basic FGF	72	61.4	(13.6-379.4)	70	64.4	(14.4-289.7)	0.202
PDGF-BB	91	444.8	(9.1-5535.9)	88	435.6	(31.3-11803.5)	0.582
VEGF	71	54.7	(13.3-648.1)	64	69.5	(12.5-359.8)	0.165
Hematopoietic factor							
G-CSF	88	123.5	(36.6-1240.3)	87	122.9	(33.7-911.1)	0.946
Interferon-γ	91	114.2	(21.2-1451.8)	88	137.5	(21.2-854.5)	0.324
TNF α	90	91.2	(22-1072.7)	84	101.7	(25.6-784.6)	0.370

POD, postoperative day; IL, interleukin; IP, interferon γ -induced protein 10; MCP, monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; G-CSF, granulocyte colony-stimulating factor; TNF, tumor necrosis factor.

the preoperative levels of IL-1 β , IL-7, IL-8, G-CSF, IFN- γ , and TNF- α were significantly increased in patients with colon cancer, while these cytokines remained low in patients with other diseases (16). The altered cytokine levels before surgery were likely to have occurred in the present study due to different states of cancer advancement and/or different types of tumors, and such preoperative differences might have consequently hampered detection of the effects of TJ-100. Another possible explanation for the wide range of cytokine levels observed in the present study is polymorphisms of genes encoding cytokines. For instance, production of IL-1 β , TNF- α , and IL-10 from peripheral blood mononuclear cells (PBMCs) obtained from pancreatic cancer patients or healthy volunteers was evidently affected by particular gene polymorphisms (14,15).

Furthermore, the relationship between gene polymorphisms of proinflammatory cytokines and their serum levels and short-term operative results have been demonstrated in the field of esophagectomy (17). Unfortunately, these two potential differences in clinical background were not addressed in the present study. At least, our findings clearly suggested that application of serum cytokines as markers for the strength of inflammation or severity of complication after PD might not be as straightforward as previously considered.

By addressing the aforementioned potential confounding factors by comparing ratios of cytokine levels on POD3 to those on POD1, we found that changes in IL-10, IL-4, IL-9, TNF- α , and PDGF-BB were significantly reduced by TJ-100 in patients without severe complications. In animal models,

Table III. Change in serum cytokine levels between POD1 and POD3 in patients without severe abdominal inflammation.

Factor	TJ-100 (n=74)			Placebo (n=70)			P-value
	(Value on POD1)/(Value on POD3)			(Value on POD1)/(Value on POD3)			
	n	Median	(Min-Max)	n	Median	(Min-Max)	
Interleukin							
IL-2	5	1.1	(0.5-1.4)	4	0.7	(0.4-1)	0.178
IL-7	73	1.3	(0.3-16.5)	70	1.2	(0.3-7.2)	0.164
IL-9	37	1.2	(0.4-6.2)	35	0.8	(0.2-3.5)	0.031
IL-13	74	1.9	(0.4-18.1)	69	1.5	(0.2-14.3)	0.177
IL-15	0	-	(0.4-18.1)	2	0.9	(0.9-0.9)	-
IL-5	74	1.5	(0.2-7.3)	70	1.1	(0.2-5.4)	0.095
GM-CSF	26	0.8	(0.1-4.5)	26	0.6	(0.2-2.8)	0.185
IL-1β	60	1.2	(0.3-7.4)	59	1	(0.3-4.9)	0.140
IL-6	73	0.3	(0-50.5)	70	0.3	(0-1)	0.785
IL-12	47	1.2	(0.3-3.8)	42	0.8	(0.2-4.3)	0.105
IL-17	39	1.2	(0.2-5.2)	34	0.8	(0.1-7)	0.094
IL-1Ra	66	1.1	(0.3-10.3)	66	1	(0.2-7.8)	0.116
IL-4	63	1.7	(0.5-7.9)	60	1.1	(0.2-7.7)	0.015
IL-10	48	1.1	(0.2-5.4)	49	0.8	(0.2-6.6)	0.032
Chemokine							
IL-8	74	0.8	(0.1-14.1)	70	0.8	(0.3-4.7)	0.444
IP-10	74	1.1	(0.4-27)	70	1.1	(0.4-12.2)	0.516
MCP-1	74	0.7	(0.3-30.1)	70	0.7	(0.2-2.7)	0.461
MIP-1α	73	1.1	(0.5-6.5)	70	1.1	(0.3-6.5)	0.230
MIP-1β	74	0.9	(0.5-3.8)	70	0.8	(0.3-2.4)	0.430
RANTES	74	0.9	(0.2-8.8)	70	0.8	(0-18.2)	0.345
Eotaxin	23	0.8	(0.4-2.1)	27	0.9	(0.2-1.9)	0.668
Growth factors							
Basic FGF	53	1.1	(0.3-12)	48	0.8	(0.1-5.9)	0.195
PDGF-BB	74	1.2	(0.1-22.8)	69	0.8	(0-16.3)	0.049
VEGF	50	1.2	(0.3-10.6)	42	1	(0.1-5.1)	0.195
Hematopoietic factor							
G-CSF	69	1.1	(0.1-93.5)	66	1	(0.2-7.5)	0.370
Interferon-γ	74	1.2	(0.2-8)	69	1.1	(0.3-6.3)	0.276
TNF-α	70	1.2	(0.3-8.6)	65	1	(0.2-4.2)	0.041

POD, postoperative day; IL, interleukin; IP, interferon γ -induced protein 10; MCP, monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; G-CSF, granulocyte colony-stimulating factor; TNF, tumor necrosis factor.

TJ-100 decreased expression of TNF- α in target organs (12,18), reducing postoperative paralytic ileus and bacterial translocation. Furthermore, TJ-100 decreased levels of serum C-reactive protein in patients following surgery for colon/rectal disease (19), suggesting that TJ-100 could ameliorate inflammatory responses. In contrast, the present analysis demonstrated a higher increase in TNF- α level from POD3 to POD1 in patients who received TJ-100 compared to the Placebo Group, indicating that TJ-100 might enhance the early inflammatory response after PD. The contradiction between previous observations and ours may be derived from the different extents of surgical stress. While the uncontrolled expression of TNF- α is associated

with an overwhelming inflammatory response, multiple-organ dysfunction, and impaired tissue recovery, extensive suppression of TNF- α results in frequent occurrence of infectious complications after surgery (20). The appropriate inflammatory response elicited against surgical stress is clearly essential for immunological defense and tissue repair, and the biological influence of sustained serum levels of TNF- α in patients receiving PD and TJ-100 should be investigated further.

IL-4, IL-10, and PDGF-BB are associated with wound healing (21-24), while increased IL-9 might enhance intestinal muscle contractility and protect against bacterial shock by modulating inflammatory and anti-inflammatory

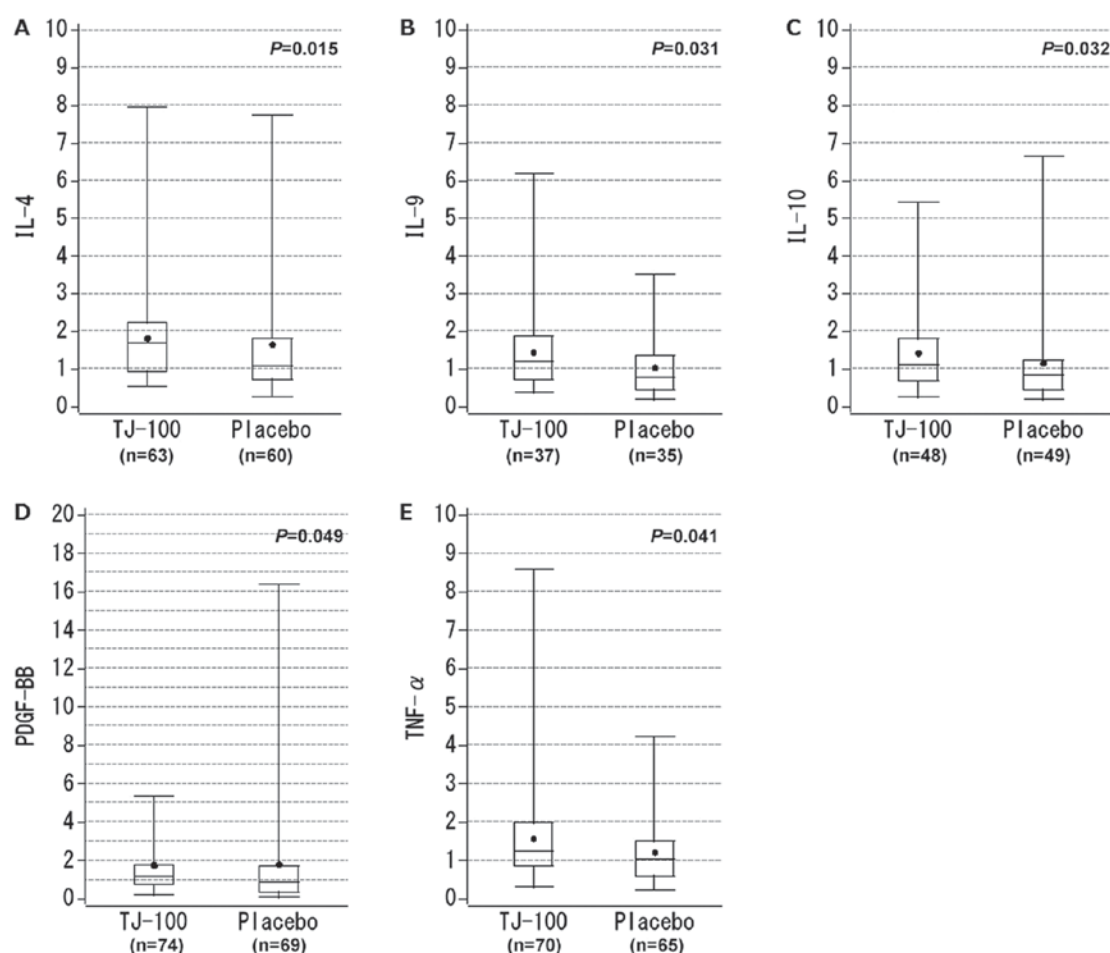


Figure 2. The ratios of cytokine levels on POD3 to those of POD1 were compared between the TJ-100 Group and the Placebo Group. A significant difference was observed in (A) IL-4, (B) IL-9, (C) IL-10, (D) PDGF-BB and (E) TNF- α . Dots represent the means and bars inside the boxes represent the medians of the ratios. IL, interleukin; TNF, tumor necrosis factor.

mediators (25,26). These observations are partially consistent with our previous clinical study of 45 patients undergoing PD (27); however, the overall clinical significance of different changes in cytokine levels due to TJ-100 administration was not determined by the present study.

Several limitations related to this analysis should be noted in addition to the lack of baseline (preoperative) cytokine levels and information of gene polymorphisms. First, the levels of cytokines were under the detection threshold in several patients, precluding these samples from the final analysis. Similarly, the number of patients with severe complications was not large enough to detect statistical differences of cytokine transitions in the present study. Finally, the level of the cytokines at the later phase of recovery, such as 7 days after operation, could have brought better understanding of the effect of TJ-100.

In conclusion, we examined the levels of 27 cytokines after PD and their transition from POD1 to POD3 in a large number of the patients enrolled in a multi-institutional, prospective trial. At initial assessment, a wide range of cytokine levels among individuals after PD was found, suggesting the difficulties of using the cytokine levels to assume the extent of surgical stress. However, oral administration of TJ-100 altered the postoperative changes in serum levels of IL-4, IL-9, IL-10, TNF- α , and PDGF-BB. With consideration of gene polymorphism and preoperative inflammatory status, studies using

TJ-100 under surgical stress should be performed to elucidate the role of different transition pattern of cytokines.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

All authors read and approved the final manuscript. HM performed data analysis, data interpretation, figure and table creation, and wrote the manuscript. KO participated in study design, study protocol creation, literature search, data collection, data analysis and data interpretation. TF participated in

study design, data collection and manuscript revision. MSO participated in study protocol creation, data analysis and figure creation. MK, SH, YK, MS, TA, YS, YA, NK, YM, JO, HE and HN participated in study design, study protocol creation, data collection and manuscript revision. JS and HY participated in study design, study protocol creation, literature search, data collection, data analysis, data interpretation, figure and table creation, and manuscript writing.

Ethics approval and consent to participate

All patients provided written informed consent prior to study inclusion. The institutional review board of each participating institute approved the trial protocol.

Patient consent for publication

All patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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