Adoptive immunotherapy for gastric cancer using zoledronate-activated killer cells: A prospective observational study

YOSHIYUKI YAMAGUCHI, YOUSUKE KATATA, FUMINORI SANO, HIROAKI TANIOKA, MAKOTO OKAWAKI, MASAHIRO YAMAMURA and TAKESHI NAGASAKA

Department of Clinical Oncology, Kawasaki Medical School and Hospital, Kurashiki, Okayama 701-0192, Japan

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Abstract. For several years, adoptive immunotherapy (AIT) has been performed using autologous zoledronate-activated killer (ZAK) cells to develop a novel modality for cancer treatment. In the current study, data from 50 patients with incurable gastric cancer were analyzed. Patients were treated with AIT using intravenous ZAK cells every 3-4 weeks in combination with chemotherapy of the physician's choice. The possible clinical benefits were subsequently examined. The median overall survival (OS) time of all patients was 7.5 months. In patients that received 5 or more rounds of treatment, the OS was 13.5 months. Additionally, the OS times of 1st, 2nd or later line chemotherapy with ZAK cell AIT were 27.3 months and 13.3 months, respectively. No objective response was observed and the disease control rate was 67.9%. No severe adverse event was recorded. Functional Assessment of Cancer Therapy-Biologic Response Modifier analysis revealed possible improvement of quality of life after ZAK cell AIT. Univariate analysis revealed a significant positive association between longer survival times and baseline lymphocyte percentages in white blood cell counts (P<0.001), serum albumin (P=0.001), C-reactive protein (P=0.006), carbohydrate antigen (CA)19-9 (P=0.010), neutrophil-lymphocyte ratio (P<0.001) and Glasgow prognostic score (GPS). Only the GPS value (P=0.024) was a significant survival marker when analyzed using the multivariate Cox proportional hazards model. Although the results cannot provide a definitive conclusion, the current suggested that ZAK cell AIT in combination with chemotherapy is safe, feasible and may be a promising treatment option for patients with incurable gastric cancer. The GPS value at baseline may be a potential biomarker for chemo-immunotherapy.

Correspondence to: Dr Yoshiyuki Yamaguchi, Department of Clinical Oncology, Kawasaki Medical School and Hospital, Matsushima 577, Kurashiki, Okayama 701-0192, Japan
E-mail: shogo@med.kawasaki-m.ac.jp

Abbreviations: AIT, adoptive immunotherapy; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; CD, cluster of differentiation; CR, complete response; DCs, dendritic cells; GPS, Glasgow prognostic score; LAK, lymphokine-activated killer; MST, median survival time; NLR, neutrophil-lymphocyte ratio; OS, overall survival; PBMC, peripheral blood mononuclear cell; PD, progressive disease; PR, partial response; QOL, quality of life; SD, stable disease; ZAK, zoledronate-activated killer

Key words: adoptive immunotherapy, zoledronate, zoledronate-activated killer cells, γδT cells, gastric cancer

Introduction

Gastric cancer is one of the most common malignancies and is the leading cause of mortality both in Japan and globally (1). Systemic chemotherapies for the disease have progressed rapidly in recent years, resulting in survival benefit to patients, although they remain unsatisfactory. To move the treatment options beyond systemic chemotherapy, novel modalities are urgently needed. To this end, we have conducted adoptive immunotherapy (AIT) trials using *ex vivo*-activated autologous lymphocytes-namely, lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes, *in vitro* tumor-sensitized lymphocytes, and tumor antigen peptide-pulsed dendritic cell-activated killer cells-although the tumor responses have remained poor (2,3). However, other researchers have achieved survival benefits in hepatocellular carcinoma patients using postoperative LAK cell transfer (4) and in lung cancer patients using LAK cell transfer in combination with chemoradiotherapy (5), suggesting that AIT may have a benefit in terms of survival rather than tumor shrinkage per se.

Following on these results, we next established a system for generating another type of effector lymphocytes, zoledronate-activated killer (ZAK) cells, which consist of natural killer (NK) cells and γδT cells (6). It has been reported that γδT cells have the ability to kill a wide variety of tumor cells, and also play an important role in the innate immune system (7). Moreover, γδT cells have been shown to possess an antigen-presenting function (8). Other researchers have described the safety and feasibility profiles of γδT cells for cancer treatment (9,10). Since 2009, we have also conducted a prospective observational study of AIT using ZAK cells for patients with various types of incurable cancer.

In this study, we analyzed a series of cumulative data from patients with advanced or metastatic gastric cancer and

demonstrated a possible survival benefit of ZAK cell AIT in combination with chemotherapy. We also identified a candidate biomarker for predicting a survival benefit from ZAK cell AIT.

Patients and methods

Study design. The data series from a prospective observational study conducted at Kawasaki Medical School Hospital between May 2009 and July 2017 was analyzed. All participating patients had a diagnosis of incurable gastric cancer with a performance status that allowed them to visit our outpatient clinic. All patients provided written informed consent. Exclusion criteria were as follows: Consecutive use of steroids or immunosuppressants, the presence of autoimmune diseases, a case that was too difficult to manage at an outpatient clinic, and/or uncontrolled complications. Participants were considered for the study until they were deceased, they withdrew their consent, or follow-up contact was lost. All aspects of patients' treatments over time, including specific chemotherapy agents and/or combinations, as well as the dose, schedule, and duration of AIT, were determined by a physician on a case-by-case basis. This prospective study was reviewed about science and ethics and approved by the Research Ethics Committee of Kawasaki Medical School and Hospital (approval no. 240, UMIN000021797).

ZAK cell generation and transfer. ZAK cell generation has been described in detail elsewhere (6). Briefly, PBMCs were obtained from the heparinized venous blood of patients by centrifugation, then stimulated with interleukin-2 plus zoledronate and cultured for 10 to 14 days. ZAK cells were harvested by centrifugation, washed twice, resuspended in 100 ml saline after filtering through a 200-μm mesh, and administered intravenously for 30 min every 3-4 weeks in a chemotherapy-off period. At each infusion, patients had blood drawn to prepare ZAK cells for the next transfer. Bacterial, endotoxin, and mycoplasma examinations were completed before each administration to make sure there was no contamination.

Clinical efficacy. Survival data of the patients were collected from patient records. If the prognosis was unknown, a letter was sent requesting this information from the doctor in charge. Objective tumor response was evaluated by computed tomographic examinations. Data were collected at baseline (before ZAK cell AIT) and every 2 to 3 months. Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were determined by the investigator according to the RECIST v1.1 criteria (11). As tumor markers, the levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9 were also measured every 2 to 3 months.

QOL analysis. Assessment of QOL was performed by Functional Assessment of Cancer Therapy-Biologic Response Modifier (FACT-BRM) analysis (12) before and after 5 administrations of ZAK cells. Documents were collected by research coordinators and analyzed independently of the physicians.

Statistics. Statistical analysis was conducted using SPSS software (IBM, Corp.). Survival curves were drawn by Kaplan-Meyer analysis to estimate the median survival time. Relationships between survival and hemato-chemical blood examination data were analyzed in a univariate setting using the log-rank test, where patients were divided into two groups, a higher group and a lower group based on the median value of each clinical measurement, then compared statistically. Multivariate analysis using the Cox proportional hazard model was also performed. Data sets of tumor markers and QOL were analyzed using the paired t-test. Values are presented as means ± standard deviations and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of patients. Fifty-eight patients with gastric cancer were treated with ZAK cell AIT but 3 patients in the postoperative adjuvant setting were excluded from the analysis. The remaining 55 patients included 31 males and 24 females with a median age of 60, ranging from 32 to 88 years. Metastatic organs included the peritoneum, liver, lymph nodes, and bone; 48 patients had at least one metastatic site and 7 had two or more metastatic organs. Positive, negative, and unknown Her2 status were observed in 4, 11, and 40 patients, respectively. First-line chemotherapy had already failed in 51 (93%) patients. Concurrent anti-cancer chemotherapy was administered in 43 patients (78%), and in most cases this consisted of S-1, taxan or both, as shown in Table I.

Feasibility of ZAK cell generation and transfer. The generation of ZAK cells was carried out 412 times in total, and 393 cultures (95.4%) were uneventful (Table II). ZAK cells contained mainly CD56+ NK cells and γδT cells at median values of 79 and 13%, ranging from 69% to 87% and 3% to 52%, respectively. ZAK cells were administered once to 4 times in 24 patients, 5 to 9 times in 16 patients, 10 to 19 times in 4 patients, 20 to 29 times in 3 patients, and 30 times or more in 3 patients; the median value was 4 times, including 5 patients who never received ZAK cell transfer because of disease progression in 4 cases and no lymphocyte growth in 1 case (Table II). The mean number of total cells transferred was 6.5×10^8 cells among all the treated patients and 12.3×10^8 cells among those treated more than 5 times. No bacteria, endotoxin, or mycoplasma was detected in any of the cultures.

Survival analysis. The overall survival (OS) of all the patients treated is displayed in Fig. 1A and B, while the survival analysis is summarized in Table III. Five patients who received no administration of ZAK cells were excluded from the analysis. With a median follow-up time of 12.3 months (range: 1.2-55.1), the median OS was 7.5 months (95% confidence interval (CI): 3.9-11.0) for all patients treated (Fig. 1A); this value increased to 13.5 months (95% CI: 8.1-18.9) when limited to patients receiving more than 5 ZAK cell AITs (Fig. 1B, solid curve), but decreased to 3.0 months (95% CI: 2.2-3.8) in the group of patients receiving less than 4 administrations (Fig. 1B, dotted curve; Table III). With respect to the combination chemotherapy, the OS times of 1st line and 2nd line or later chemotherapy plus 5 or more administrations of ZAK cell AIT were 27.3 months (95% CI: 7.7-45.0) and 13.3 months (95% CI: 9.4-17.3), respectively, compared to an OS of 8.0 months in patients who had already finished the 1st line chemotherapy

Table I. Patients enrolled in the ZAK cell AIT trial.

Variable	N (%)
Total no.	55
Male/female	31/24
Age (median, range)	60, 32-88
Target and metastatic organs	
Peritoneum	27 (49)
Liver	16 (29)
Lymph node	14 (25)
Bone	3 (5)
Others	3 (5)
Organs affected	
1	48 (87)
≥2	7 (13)
Her2 status	
Positive	4 (7)
Negative	11 (20)
Uknown	40 (73)
Number of previous regimens	
0	4 (7)
≥1	51 (93)
Concurrent treatments	
Chemotherapy	43 (78)
S-1	10 (18)
S-1+CDDP	9 (16)
PTX	9 (16)
S1+PTX	4 (7)
CPT-11	3 (5)
DTX	2 (4)
Others	6 (11)
None	12 (22)

ZAK, zoledronate-activated killer; AIT, adoptive immunotherapy; PTX, paclitaxel; DTX, docetaxel; S-1, Tegafur/Gimeracil/Oteracil; CDDP, cisplatin; PTX, paclitaxel; CPT-11, irinotecan.

Table II. Feasibility, quality and outcome of the transfer of ZAK cell adoptive immunotherapy.

Variable	Value
Total culture no.	412
Success of culture, n (%)	393 (95.4%)
ZAK cell phenotype, mean, range	
CD3	37, 20-68
γδT	13, 3-52
CD56	79, 69-87
No. of administrations	
0	5[a]
1-4	24
5-9	16
10-19	4
20-29	3
≥30	3
Median (range)	4 (0-44)
Mean ± SD	7.0±9.3
Total cell no. administered, mean	
All patients treated	$6.5x10^8$
Patients treated ≥5 times	$12.3x10^8$
Contamination detected	0
Endotoxin >4.0 pg/ml	0

[a]The number of the patients who could not receive ZAK cell adoptive immunotherapy due to disease progression (n=4) and no lymphocyte growth (n=1). ZAK, zoledronate-activated killer.

and received 5 or more administrations of ZAK cell AIT as monotherapy (Table III).

Tumor response. Tumor response is shown in Table IV. Of all the patients treated, 28 were evaluable for objective tumor responses. No CR or PR was observed. Nineteen patients (67.9%) showed SD status, and thus the disease control rate was estimated as 67.9%. Changes in tumor markers were analyzed in 26 patients who received 5 or more administrations of ZAK cell AIT. Although the values of CEA and CA19-9 decreased in 9 and 7 patients, respectively, the mean values of each marker increased with no significant difference (Table V).

Adverse events. Of 50 patients treated, 1 showed temporary low-grade fatigue (grade 1) after ZAK cell transfer. No other adverse events higher than grade 2 related to ZAK cell administration were experienced in any of the patients treated.

QOL analysis. The results of QOL analysis of 24 assessable data sets are shown in Table VI. The score of functional well-being was significantly improved after ZAK cell transfer (P=0.024). Moreover, the total FACT-General and FACT-BRM scores showed trends of improvement after ZAK cell AIT, although these improvements were not statistically significant (P=0.057 and 0.073, respectively).

Analysis of patients with survival benefit. The relationships between the survival and clinical measurements at baseline were analyzed in an attempt to identify biomarkers of ZAK cell AIT benefit in patients with incurable gastric cancer. In the univariate analysis, there was a significant difference in several clinical measurements: Better survival was observed in the groups with a lymphocyte percentage ≧28 in the white blood cell count (P<0.001; Fig. 2, solid curve), serum albumin ≧3.6 (P=0.001), serum C-reactive protein (CRP) <0.17 (P=0.006), serum carbohydrate antigen (CA)19-9 <40.2 (P=0.010), and neutrophil-lymphocyte ratio (NLR) <2.3 (P<0.001), and in the GPS0 (P<0.001) and GPS1 (P=0.042) groups compared with the GPS2 group (Table VII). A representative comparison of the Kaplan-Meier curves of the baseline lymphocyte percentage is shown in Fig. 2. In the multivariate analysis, GPS was found to be associated with survival: Significantly better survival was observed in the low GPS group with an HR of 3.201 (95% CI, 1.165-8.791; P=0.024; Table VIII).

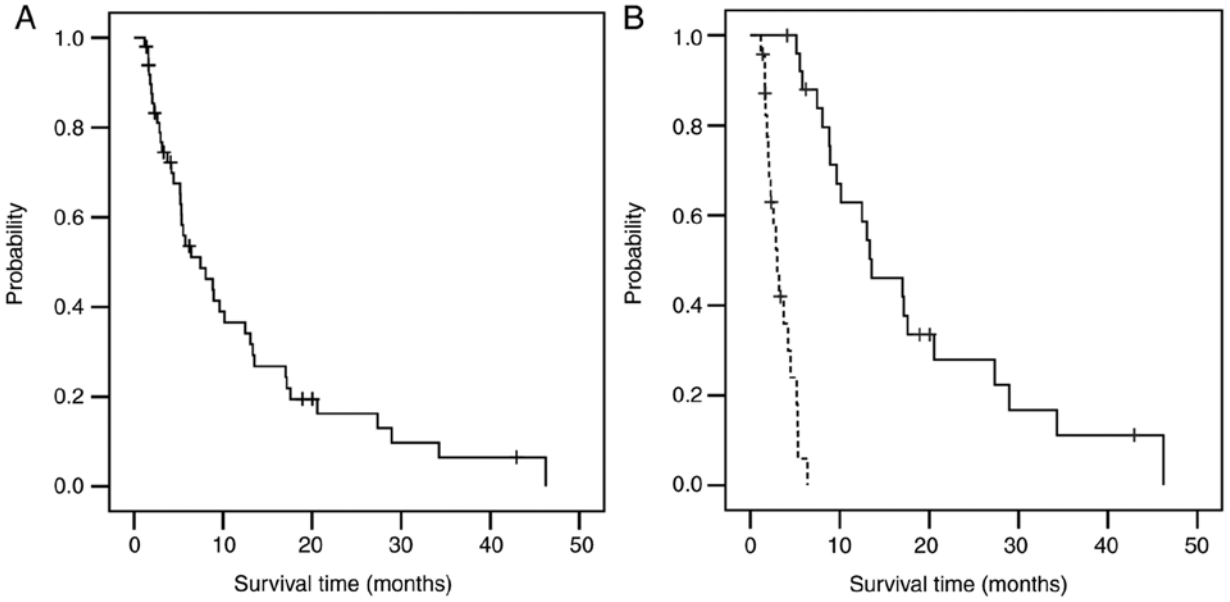

Figure 1. Analysis of OS. Kaplan-Meyer analysis was performed to estimate median survival time. (A) The OS times of enrolled patients. Median OS was 7.5 months (95% CI, 3.9-11.0 months) and median follow-up time was 12.3 months (range, 1.2-55.1 months). (B) OS of patients treated with ZAK cell AIT ≥5 (solid curve). The median OS was 13.5 months (95% CI, 8.1-18.9 months). The median OS of patients treated with ZAK cell AIT ≤4 (dotted curve) was 3.0 months (95% CI, 2.2-3.8 months). OS, overall survival; CI, confidence interval; ZAK, zoledronate-activated killer.

Table III. OS of ZAK cell adoptive immunotherapy.

Therapy	No. of patients	MST (months)	95% CI (months)
All patients	50	7.5	3.4-11.5
≤4 times	24	3.0	2.2-3.8
≥5 times	26	13.5	8.1-18.9
Combination	40	9.6	2.9-16.3
≤4 times	18	4.2	2.6-5.8
≥5 times	22	17.0	11.3-22.7
1st line	11	17.2	0.5-33.8
≤4 times	3	4.5	-
≥5 times	8	27.3	7.7-45.0
≥2nd line	29	7.5	2.2-12.7
≤4 times	15	3.7	2.6-4.9
≥5 times	14	13.3	9.4-17.3
ZAK alone	10	2.3	1.5-3.1
≤4 times	6	2.0	1.7-2.2
≥5 times	4	8.0	4.4-11.7

Median follow-up time was 12.3 months (range, 1.2-55.1 months). OS, overall survival; ZAK, zoledronate-activated killer; CI, confidence interval; MST, mean survival time.

Table IV. Objective responses of patients treated with ZAK cell adoptive immunotherapy.

Response	No. of patients (%)
CR	0 (0)
PR	0 (0)
SD	19 (67.9)
PD	9 (32.1)
DCR (CR+PR+SD)	19 (67.9)

ZAK, zoledronate-activated killer; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; DCR, disease control rate.

Discussion

We have been conducting an observational study of AIT using ZAK cells for the treatment of patients with incurable cancer since 2009. In this series, we have already reported a possible survival benefit of ZAK cell AIT in combination with chemotherapy in patients with pancreatic cancer (13). In this paper, we analyzed patients with gastric cancer. Our analysis showed that, over approximately 400 ZAK cell generations in gastric cancer patients, 95% of cultures were uneventful with no contamination, indicating that our system had good feasibility for the preparation of ZAK cells for gastric cancer patients, just as it was previously shown to have good feasibility for the preparation of ZAK cells for pancreatic cancer patients (13). ZAK cells from gastric cancer patients also showed a heterogenous phenotype consisting of NK cells and γδT cells in our ZAK cell generation system, although γδT cells accounted for a lower percentage of total ZAK cells in gastric cancer patients compared to pancreatic cancer patients (13% vs. 45%, respectively) (13), suggesting that the ability to generate ZAK cells may differ among cancer types.

The survival analysis showed that although the median OS was 7.5 months in all patients after our ZAK cell AIT, the OS was prolonged to 13.5 months in patients who received ZAK

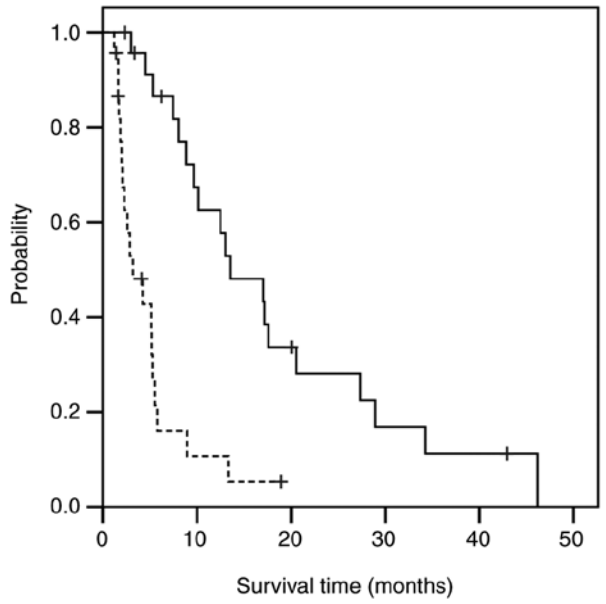

Figure 2. Univariate analysis of survival and the percentage of peripheral blood lymphocytes at baseline ZAK cell adoptive immunotherapy. There was a significant difference in the survival curves between patients with lymphocyte percentage ≥28 (solid curve) and those with a lymphocyte percentage <28 (dotted curve) according to the log-rank test (P<0.001). ZAK, zoledronate-activated killer.

Table V. Changes of tumor markers in patients treated with ZAK cell adoptive immunotherapy.

Tumor marker, no.	Value (mean ± SD)	P-value
CEA, 23[a]		
Baseline	18.6±41.9	0.1001
After ZAK	50.8±96.5	
CA19-9, 23[b]		
Baseline	1,557.9±4,192.3	0.6107
After ZAK	2,560.5±10,051.4	

[a]CEA decreased in 9 patients. [b]CA19-9 decreased in 7 patients. ZAK, zoledronate-activated killer; CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

cell AIT 5 times or more, while the OS in those who received ZAK cell AIT fewer than 4 times was very poor. The OS times were extended further, to 27.3 and 13.3 months, when ZAK cell AIT was performed 5 times or more in combination with 1st line and 2nd line or later chemotherapy, respectively. It was reported that the median OS was 13.0 months in patients assigned to S-1 plus cisplatin treatment in the SPIRITS study, which is a pivotal phase III trial of 1st line chemotherapy for advanced or refractory gastric cancer patients (14). The START study also reported a similar OS of 12.5 months in gastric cancer patients treated with docetaxel plus S-1 (15). Moreover, the RAINBOW study, which is a randomized, placebo-controlled, double-blind, phase 3 trial for advanced gastric cancer in a 2nd line setting, showed an OS of 9.6 months in the ramucirumab plus paclitaxel group, which was significantly longer than that of 7.4 months in the placebo plus paclitaxel group (16). Qiao *et al* (17), have indicated the benefit of the combination of dendritic cell-cytokine-induced killer (DC-CIK) cell immunotherapy over S-1 plus cisplatin chemotherapy in advanced gastric cancer, and they reported that the DC-CIK infusions demonstrated a preferable disease control rate (CR+PR+SD) of 76.9% in the DC-CIK combined with the S-1 plus cisplatin group compared with that of 47.1% in the S-1 plus cisplatin group. Therefor, our OS times of 13.5, 27.3 and 13.3 months in patients who received ZAK cell AIT 5 times or more and our disease control rate of 67.9% would seem to constitute a favorable result, although ZAK cell AIT alone had only a marginal effect. ZAK cell AIT was well tolerated with no serious adverse events, and the FACT-BRM analysis revealed a possible improvement of QOL after ZAK cell AIT. Taken together, these results suggest that our ZAK cell AIT in combination with chemotherapy might be a promising treatment option for patients with incurable gastric cancer, as well as for patients with pancreatic cancer as shown in our previous study (13).

What is a mechanism by which ZAK cell AIT extends the benefits of chemotherapy? Kono *et al* (18), performed AIT with tumor-associated lymphocytes in patients with stage IV gastric or colon cancer and indicated that the expression of TCR zeta chains, which were made up of T-cell receptor-CD3-associated signal transducing molecules, was further down-regulated in correspondence with disease progression in the individual patients, and that AIT could induce increased or stable TCR zeta expression, indicating the significance of the addition of AIT in treating gastric cancer. More recently, an anti-programmed death-1 (-PD-1) antibody, nivolumab, showed a clear survival benefit for patients with previously treated gastric cancer (19). This indicates that the host immune system does respond to cancer cells and that approaches which involve the host immune system are important in the treatment of gastric cancer. Interestingly, Iwasaki *et al* (20) demonstrated the possible involvement of PD-1-PD-ligand 1 (PD-L1) interaction in the negative regulation of γδT cells for cytokine production and cytotoxic activity. Zhao *et al* (21), developed chimeric antigen receptor-modified T (CAR-T) cells bi-specific for tumor antigen Trop2 and PD-L1 and showed that Trop2/PD-L1 CAR-T cells were able to target Trop2/PD-L1 and checkpoint blockade, and also had a killing effect on gastric cancer, resulting in an improvement of the killing effect of CAR-T cells. These findings suggest the exciting possibility of using ZAK cell AIT combined with acti-PD-1/PD-L1 antibody for the treatment of gastric cancer.

In contrast to the possible survival benefits, the objective tumor response to the treatment with ZAK cell AIT and chemotherapy was minimal. One possible explanation for this is that about 80% of the patients in this observational study underwent treatment in a 2nd line or later chemotherapy setting. Another possible explanation is that the poor tumor response was due to an inherent property of immunotherapy. In a vaccine trial of sipuleucel-T, only 1 of more than 300 patients with prostatic cancer showed an objective tumor response (22), suggesting that immunotherapy may provide a survival benefit without inducing an objective tumor response. More attention should be paid to this property of cancer immunotherapy.

We also sought adequate biomarkers to identify gastric cancer patients suitable for ZAK cell transfers. We initially

Table VI. QOL analysis.

Subscale	QOL points at baseline (mean ± SD)	QOL points after AIT (mean ± SD)	Improvement (points)	95% CI of improvement	P-value
Physical well-being	20.6±4.8	20.9±4.9	0.3	-2.96-3.62	0.828
Social well-being	20.0±5.9	21.1±5.2	1.0	-2.48-4.56	0.528
Emotional well-being	13.7±5.8	16.5±5.2	2.8	-0.84-6.51	0.118
Functional well-being	15.3±6.3	19.3±6.3	4.0	0.65-7.35	0.024
BRM physical	19.7±4.0	20.8±2.6	1.2	-1.62-3.93	0.379
BRM cognitive/emotional	14.1±5.7	16.6±5.2	2.5	-0.56-5.56	0.100
FACT-BRM TOI	69.6±18.1	77.6±16.2	8.0	-2.56-18.53	0.124
FACT-general total score	69.5±18.6	77.7±17.0	8.2	-0.29-16.71	0.057
FACT-BRM total score	103.3±25.4	115.2±23.5	11.9	-1.32-25.04	0.073

QOL points were assessed by FACT-BRM and calculated at baseline and after zoledronate-activated killer cell AIT. Differences between those points were indicated as the improvement. QOL, quality of life; CI, confidence interval; BRM, biological response modifier; FACT, Functional Assessment of Cancer Therapy; TOI, trial outcome index; AIT, adoptive immunotherapy.

Table VII. Univariate survival analysis using log-rank test on baseline biochemical measures in ZAK cell adoptive immunotherapy.

Parameter	n	MST	95% CI	P-value
Lymphocyte (%)				
<28	23	3.2	0.9-5.5	<0.001
≥28	24	13.5	6.8-20.3	
Lymphocyte count (/μl)				
<1,345	23	5.2	1.8-8.6	0.093
≥1,345	23	8.9	5.8-12.1	
Albumin (g/dl)				
<3.6	22	4.2	1.4-7.0	0.001
≥3.6	25	12.5	6.7-18.3	
CRP (mg/dl)				
<0.17	14	17.2	10.4-23.9	0.006
≥0.17	15	5.3	3.6-7.0	
CEA (ng/ml)				
<6.8	22	12.5	6.8-18.1	0.093
≥6.8	23	5.2	3.3-7.0	
CA19-9 (U/ml)				
<40.2	22	13.3	11.8-14.9	0.010
≥40.2	23	5.2	3.6-6.7	
NLR				
<2.3	23	17.0	11.1-23.0	<0.001
≥2.3	23	3.2	0.9-5.5	
GPS				
0	17	13.0	1.0-25.1	0.065 for GPS (1), <0.001 for GPS (2)
1	9	5.8	3.7-7.8	0.042 for GPS (2)
2	3	2.0	-	

ZAK, zoledronate-activated killer; MST, median survival time; CI, confidence interval; CRP, C-reactive protein; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; NLR, neutrophil-lymphocyte ratio; GPS, Glasgow prognostic score.

Table VIII. Multivariable Cox regression analysis between the survival and biochemical measurement at baseline of ZAK cell adoptive immunotherapy.

Parameter	HR	95% CI	P-value
Lymphocyte (%)	1.021	0.889-1.172	0.772
Lymphocyte count (μl)	1.000	0.998-1.002	0.862
Albumin (mg/dl)	1.821	0.050-66.162	0.744
CRP (mg/dl)	1.387	0.666-2.891	0.382
CEA (ng/ml)	1.000	1.000-1.001	0.198
CA19-9 (lU/ml)	1.000	1.000-1.000	0.104
NLR	0.867	0.643-1.169	0.350
GPS	3.201	1.165-8.791	0.024

ZAK, zoledronate-activated killer; HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; NLR, neutrophil-lymphocyte ratio; GPS, Glasgow prognostic score.

had an interest in the Her2 status, which may influence the efficacy of ZAK cell AIT. However, in the end we decided to remove Her2 status from the analysis, because only limited data were available and there was no clear difference in survival between Her2-positive and -negative patients (data not shown). Univariate analysis showed significantly longer survival in patients having a baseline lymphocyte percentage in white blood cells of ≧28%, serum albumin≧3.6, CRP<0.17, CA19-9<40.2, NLR value<2.2, or a GPS of 0 or 1. However, only the GPS value was shown to be a significant survival marker in this trial when analyzed using a multivariate Cox proportional hazards model. Hirahara *et al* demonstrated that the NLR-platelet-lymphocyte ratio might be a promising marker for predicting tumor response and prognosis in the chemotherapy of patients with advanced gastric cancer (23). Yuan *et al* indicated that CA19-9, palliative gastrectomy, first-line chemotherapy, and GPS are the prognostic factors that predict OS when treating patients with advanced gastric cancer (24). Taken together, these results suggest that the lymphocyte percentage in white blood cells, serum albumin, CRP, CA19-9, NLR, and GPS levels at baseline may be possible biomarkers not only for chemotherapy but also for ZAK cell AIT in patients with incurable gastric cancer. A large-scale prospective study is necessary to fully investigate this possibility.

This study has some limitations: There may have been selection biases in the observational study, different numbers of patients were analyzed in the individual analyses, and the data sets were incomplete for some of the analyses. Any of these could have led to a misinterpretation of the study results. We plan to clear up these limitations by conducting a next phase II trial for ZAK cell AIT, plans for which are already underway.

In summary, although it is too early for a definitive conclusion, ZAK cell AIT in combination with chemotherapy is safe and feasible and might be a promising treatment option for patients with incurable gastric cancer. The baseline value of GPS is a candidate biomarker for this chemo-immunotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YY, YK and MO designed the current study and performed ZAK cell AIT. FS monitored the quality of ZAK cells and study progress. HT, MY and TN analyzed clinical data. YY wrote the manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Research Ethics Committee of Kawasaki Medical School and Hospital (approval no. 240, UMIN000021797). Written informed consent was obtained from all participants.

Patient consent for publication

Patient consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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