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Association of Probiotic *Clostridium butyricum* Therapy with Survival and Response to Immune Checkpoint Blockade in Patients with Lung Cancer

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ABSTRACT

Gut dysbiosis caused by antibiotics impairs response to immune checkpoint blockade (ICB). Gut microbiota is becoming an attractive therapeutic target for cancer. The *Clostridium butyricum* MIYAIRI 588 strain is a probiotic therapy used to improve symptoms related to antibiotic-induced dysbiosis in Japan. We hypothesized that probiotic *Clostridium butyricum* therapy (CBT) may affect the therapeutic efficacy of ICBs. We retrospectively evaluated 118 patients with advanced non–small cell lung cancer treated with ICBs at Kumamoto University Hospital (Kumamoto-shi, Kumamoto, Japan). Survival analysis comparing patients given CBT before and/or after ICB was conducted using univariate analyses and Cox proportional hazards regression models using propensity score. Propensity score analyses confirmed that probiotic CBT significantly prolonged progression-free survival (PFS) and overall survival (OS). Probiotic CBT significantly associated with longer PFS and OS even in patients who received antibiotic therapy. This study suggests that probiotic CBT may have a positive impact on therapeutic efficacy of ICB in patients with cancer.

Introduction

Immune checkpoint blockade (ICB) has led to a paradigm shift in cancer therapy, yet clinical benefit from ICB is restricted to only a proportion of patients (1–4). The gut microbiota play a role in the response and resistance to immunotherapy (2, 4–6). Gut dysbiosis caused by antibiotics impairs response to ICB, suggesting that an intact gut microbiota is essential to improve the efficacy of ICB and an attractive therapeutic target for cancer treatment (1, 3, 4, 7). Manipulating commensal microbiota enhances the efficacy of ICB in murine models (2, 4, 5). However, the clinical value of modulating gut microbiota by administration of specific bacterial species in patients with cancer receiving ICB remains largely unknown (2).

Clostridium butyricum is a spore-forming bacillus named for its capacity to produce high amounts of butyric acid and is found in soil. *Clostridium butyricum* MIYAIRI 588 strain (MIYA-BM) is widely used as probiotic therapy to improve symptoms related to dysbiosis such as constipation, nonantimicrobial diarrhea, and antimicrobial-associated diarrhea in Japan and China (8–12). *Clostridium butyricum* increases beneficial bacteria, especially lactobacilli and bifidobacteria (9, 13–15). *Bifidobacterium* promotes antitumor immunity and facilitates efficacy of ICB (5, 6). Thus, we hypothesized that probiotic *Clostridium butyricum* therapy (CBT) may enhance the therapeutic efficacy of ICB through the modulation of gut microbiota.

Here, we found that probiotic CBT compared with no probiotic CBT significantly improved progression-free survival (PFS) and overall survival (OS) in patients with non–small cell lung cancer (NSCLC) treated with ICB. PFS and OS were significantly improved in patients treated with probiotic CBT than those not treated with probiotic CBT even in patients who received antibiotic therapy prior to ICB. These findings suggest that manipulating commensal microbiota by probiotic CBT has the potential to enhance the efficacy of ICB and probiotic CBT may improve the diminished efficacy of ICB during antibiotic treatment.

Materials and Methods

Patients

We retrospectively evaluated 118 patients with advanced NSCLC consecutively treated with ICB therapy in routine clinical practice at Kumamoto University Hospital (Kumamoto-shi, Kumamoto, Japan) between January 1, 2016, and May 31, 2019. The medical records of patients who had received nivolumab (3 mg/kg or 240 mg every 2 weeks), pembrolizumab (200 mg every 3 weeks), or atezolizumab (1,200 mg every 3 weeks) were reviewed. Treatments were provided until disease progression, unacceptable toxicity, or consent withdrawal. All patients enrolled in this study were Japanese.

To investigate whether probiotic CBT (Miyarisan Pharmaceutical Co., Ltd.) before and/or during immune checkpoint inhibitor (ICI) therapy affected PFS, OS, and response to ICI therapy in patients with NSCLC treated with ICB, those receiving probiotic CBT within 6 months before beginning ICB and/or concurrently with ICB until cessation were compared with those who did not. The history of probiotic CBT was extracted by using prescription database and also manually checked from medical records. Attending physician and/or pharmacists confirmed that all patients had taken MIYA-BM as prescribed. Patient characteristics are summarized in **Table 1**. Tumor responses of 108 patients were objectively assessed by pulmonary physicians according to RECIST, version 1.1. Diarrhea and immune-related enterocolitis were graded using the NCI Common

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Table 1. Baseline characteristics of 118 patients with advanced NSCLC receiving anti–PD-1/PD-L1 blockade.

	Probiotic CBT *N* = 39	No probiotic CBT *N* = 79	*P*
Median age (range)	68.0 (62.0–71.0)	67.0 (60.0–72.0)	0.83
Sex, *N* (%)			
Male	33 (85%)	66 (84%)	1.00
Female	6 (15%)	13 (16%)	
ECOG performance status, *N* (%)			
0	10 (26%)	23 (29%)	0.07
1	15 (38%)	45 (57%)	
2	11 (28%)	11 (14%)	
3	2 (5%)	0 (0%)	
4	1 (3%)	0 (0%)	
Body weight, median (range)	61.9 (55.6–68.7)	59.4 (51.4–68.5)	0.24
Smoking history, *N* (%)			
Current	5 (13%)	9 (11%)	0.77
Former	30 (77%)	57 (72%)	
Never	4 (10%)	13 (17%)	
Stage at initial diagnosis, *N* (%)			
I–III	16 (41%)	32 (41%)	1.00
IV	23 (59%)	47 (59%)	
Histology, *N* (%)			
Adenocarcinoma	25 (64%)	56 (71%)	0.53
Squamous/NOS	14 (36%)	23 (29%)	
EGFR mutation status, *N* (%)			
Wild-type	25 (64%)	64 (81%)	0.10
Mutant	3 (8%)	3 (4%)	
Unknown	11 (28%)	12 (15%)	
PD-L1 status, *N* (%)			
TPS ≥50%	16 (41%)	24 (30%)	0.019
TPS 1%–49%	5 (13%)	14 (18%)	
TPS <1%	14 (36%)	15 (19%)	
Unknown/undeterminable	4 (10.5%)	26 (33%)	
ICB therapy line, *N* (%)			
1st line	14 (36%)	23 (29%)	0.33
2nd line	16 (41%)	27 (34%)	
≥3rd line	9 (23%)	29 (37%)	
ICB, *N* (%)			
Nivolumab	12 (31%)	39 (49%)	0.038
Pembrolizumab	20 (51%)	36 (46%)	
Atezolizumab	7 (18%)	4 (5%)	
ICB monotherapy/combination therapy, *N* (%)			
Monotherapy	33 (85%)	74 (94%)	0.18
Combination therapy	6 (15%)	5 (6%)	
Antibiotic use within 60 days before the start of ICB therapy, *N* (%)	22 (56%)	24 (30%)	0.009
Time point of administration of probiotic MIYA-BM, *N* (%)			
Before ICI initiation	9 (23%)	—	
During ICI therapy	12 (31%)	—	
Before and during ICI therapy	18 (46%)	—	
Response to ICB, *N* (%)	*N* = 37	*N* = 69	
CR	3 (8%)	1 (1%)	0.08
PR	15 (40%)	17 (25%)	
SD	11 (30%)	31 (45%)	
PD	8 (22%)	20 (29%)	
ORR	49%	26%	
DCR	78%	71%	

Note: Pembrolizumab/pemetrexed/platinum ($n = 6$), pembrolizumab/nab-paclitaxel/carboplatin ($n = 4$), and atezolizumab/bevacizumab/carboplatin/paclitaxel ($n = 1$) were used as combination therapies with ICB and chemotherapies. Tumor response to therapy was objectively assessed by pulmonary physicians according to RECIST, version 1.1.

Abbreviations: CR, complete response; DCR, disease control rate; NOS, not otherwise specified; ORR, objective response rate; PD, progression disease; PR, partial response; SD, stable disease; TPS, tumor proportion score.

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Terminology Criteria for Adverse Events, version 5.0. This study was conducted in accordance with the amended Declaration of Helsinki. This study was performed after approval by the Kumamoto University Institutional Review Board (Kumamoto-shi, Kumamoto, Japan, IRB number, 1825; approval date, October 24, 2019), which also waived the need to obtain informed consent because the data were analyzed retrospectively and anonymously.

Statistical analysis

We presented patient characteristics as medians as appropriate. Patient characteristics were compared using Fisher exact test for categorical data and Wilcoxon rank sum test for continuous data. The Kaplan–Meier method was used to obtain estimates of PFS and OS. We compared the curves with a two-tailed log-rank test. PFS was measured from the date ICB started to the date of documented progression or death. Patients who were alive and not known to have progressed were censored. OS was measured from the date ICB started to the date of death or last follow-up. The data cutoff date was October 1, 2019. Survival analysis was conducted using univariate analyses and Cox proportional hazards regression models using propensity score to correct for potential confounding factors that may affect the treatment assignment. For multivariate modeling, we used propensity score adjustment for sex, age, body weight, Eastern Cooperative Oncology Group (ECOG) performance status, histology, smoking history, programmed cell death-ligand 1 (PD-L1) status, initial stage, ICI therapy line, ICI monotherapy/combination therapies, antibiotic use within the 60 days before the start of ICI therapy, and immune-related enterocolitis. Each factor was categorized as shown in **Table 1**; Supplementary Table S1. The method of propensity score adjustment preserved statistical power by reducing covariates into a single variable. To evaluate the adjusted effect of probiotic CBT, propensity score was estimated through a binary logistic regression providing the predicted probability with making probiotic CBT have a function above background factors. Next, we performed survival analyses using Cox proportional hazard models with inverse probability of treatment weighting (IPTW) using the propensity score that balances the relevant characteristics between probiotic CBT group versus no probiotic CBT group. To confirm the statistical robustness, we performed another method using the propensity score as covariate in Cox proportional hazard models. Statistical analyses were performed with R version 3.5.3 (The R Foundation for Statistical Computing). Statistical significance was indicated by $P < 0.05$.

Results

Patient characteristics

A total of 99 men and 19 women [median (range) age, 68 (37–83) years] with advanced NSCLC were included in this study, most patients had a performance status of 0 to 1 [93 (79%)], and patients had received anti–programmed cell death 1 (PD-1)/PD-L1 ICB as first- or second-line therapy [80 (68%)]. Thirty-nine of 118 patients (33%) received probiotic CBT within 6 months before beginning ICI and/or concurrently with ICI (**Table 1**). Among the 39 patients, 9 (23%) received probiotic CBT within 6 months before beginning ICB, 12 (31%) received concurrently with ICB, and 18 (46%) received before and during ICB therapy (**Table 1**). Probiotic CBT was administered to improve symptoms of constipation, nonantimicrobial diarrhea, or antimicrobial-associated diarrhea. The indications and characteristics of probiotic CBT are shown in Supplementary Table S2.

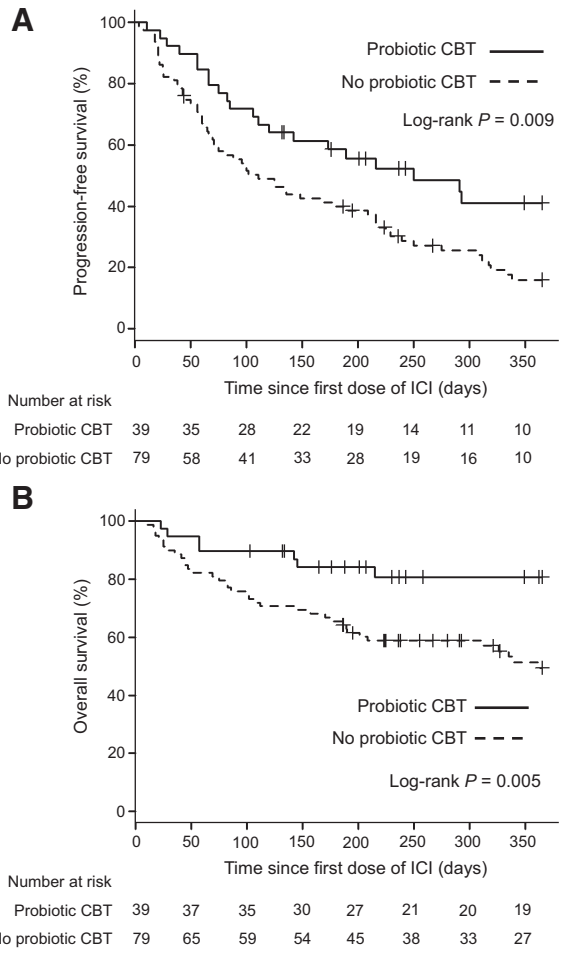

Figure 1.

Association between probiotic CBT and survival in patients with NSCLC treated with ICB. **A,** PFS in patients with NSCLC treated with ICB, stratified by administration of probiotic CBT is shown. **B,** OS in patients with NSCLC treated with ICB, stratified by administration of probiotic CBT is shown.

Probiotic CBT enhanced the efficacy of ICB in patients with NSCLC

Univariate survival analyses confirmed that probiotic CBT compared with no probiotic CBT associated with longer PFS (median, 250 vs. 101 days; $P = 0.009$) and OS [median, not reached (NR) vs. 361 days; $P = 0.005$; **Fig. 1**]. We applied Cox proportional hazard models with IPTW and propensity score was estimated by a logistic regression model. Sex, age, ECOG performance status, histology, smoking history, PD-L1 status, initial stage, ICB therapy line, ICB monotherapy/combination therapies, antibiotic use, and immune-related enterocolitis were used as the background factors (**Table 1**; Supplementary Table S1). The propensity score analysis confirmed that probiotic CBT compared with no probiotic CBT prolonged PFS [median, 250 vs. 111 days; HR, 0.37; $P = 0.001$, 95% confidence interval (CI), 0.21–0.65] and OS (median, NR vs. 361 days; HR, 0.2; $P < 0.001$; 95% CI, 0.07–0.44). To confirm statistical robustness, we performed another method using the propensity score as covariate in Cox proportional hazards regression models, which confirmed that probiotic CBT was independently associated with longer PFS (HR,

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Table 2. Characteristics of antibiotic therapy within 60 days before the start of ICB therapy in 118 patients with advanced NSCLC receiving anti–PD-1/PD-L1 blockade.

Antibiotic class	Probiotic CBT ($N = 39$)		No probiotic CBT ($N = 79$)	
	Antibiotic therapy $n = 22$ (56%)	No antibiotic therapy $n = 17$ (44%)	Antibiotic therapy $n = 24$ (30%)	No antibiotic therapy $n = 55$ (70%)
β-lactams ± β-lactamase inhibitors	6	—	6	—
Carbapenems	1	—	—	—
Macrolides	1	—	2	—
Quinolones	9	—	14	—
Quinolones + β-lactams ± β-lactamase inhibitors	3	—	—	—
Quinolones + glycopeptide + β-lactams + β-lactamase inhibitors	1	—	—	—
Quinolones + sulfonamides	—	—	1	—
Tetracyclines	—	—	1	—
Sulfonamides	1	—	—	—
Indication for antibiotic therapy	$N = 22$		$N = 24$	
Respiratory tract infection	12		9	
Gastrointestinal infection	2		0	
Skin/soft-tissue infection	2		1	
Unclear source	6		14	

0.41; $P = 0.002$; 95% CI, 0.23–0.71) and OS (HR, 0.27; $P = 0.004$; 95% CI, 0.11–0.66).

Among the 118 patients who received ICB therapy, 5 patients (4.2%) developed endoscopically confirmed immune-related enterocolitis and 34 patients (28.8%) developed diarrhea during ICB therapy (Supplementary Table S1). Immune-related adverse events associate with efficacy of ICB in NSCLC (16). Both immune-related enterocolitis and diarrhea during ICB therapy associated with improved survival outcomes (17). In our cohort, 12 patients (31%) among 39 patients who received probiotic CBT developed diarrhea during ICB therapy (Supplementary Table S1). Probiotic CBT was started to improve diarrhea during ICI therapy for 6 patients and diarrhea due to endoscopically confirmed immune-related enterocolitis for 1 patient (Supplementary Table S2). Thus, we considered not only immune-related enterocolitis, but also diarrhea during ICB therapy as confounding factors. As the result of survival analyses using Cox proportional hazards regression models using propensity score, we confirmed that probiotic CBT was independently associated with improved survival outcomes (PFS: HR, 0.37; $P = 0.001$; 95% CI, 0.21–0.68 and OS: HR, 0.20; $P < 0.001$; 95% CI, 0.08–0.50; Supplementary Table S3).

Probiotic CBT improved ICB efficacy in antibiotic-treated patients

Antibiotic therapy before the start of ICB therapy reduces response to ICB (1, 3, 4, 7). However, no treatment which can improve the patients' survival deteriorated by antibiotics are available. We hypothesized that probiotic CBT would improve the response to ICB even in patients who had received antibiotics prior to ICB. In this study, 46 of 118 patients (39%) received antibiotic therapy within 60 days before the start of ICI therapy. Twenty-two of 39 patients (56%) received antibiotic therapy in probiotic CBT group. Twenty-four of 79 patients (30%) received antibiotic therapy in no probiotic CBT group. The indications and characteristics of antibiotic therapy within 60 days before the start of ICI therapy are shown in **Table 2**. β-lactam– and quinolone-based antibiotic therapy were the most common antibiotics used for both groups.

First, we evaluated the association of antibiotic therapy within 60 days before the start of ICB therapy with survival in patients with NSCLC. In contrast to the results reported previously, antibiotic therapy prior to initiation of ICB was not significantly associated with worse clinical outcomes with ICB therapy in the total 118 patient cohort (Supplementary Fig. S1). Next, we evaluated the impact of probiotic CBT on survival in those who received or those who did not receive antibiotics within 60 days of ICI therapy. In patients with no antibiotic therapy, probiotic CBT did not improve PFS ($P = 0.18$), but probiotic CBT was associated with improved OS (median OS, NR vs. NR; $P = 0.039$; **Fig. 2A**). Patients who received antibiotic therapy had improved PFS and OS when given probiotic CBT compared with those not given probiotic CBT (median PFS, 216 vs. 71 days; $P = 0.003$; median OS, NR vs. 182 days; $P = 0.02$; **Fig. 2B**).

Discussion

This study reports the potential positive impact of probiotic CBT on ICB efficacy in patients with lung cancer. Probiotic CBT was associated with favorable clinical outcomes in patients with NSCLC treated with ICB. In addition, probiotic CBT was significantly associated with improved therapeutic outcomes in patients who received antibiotic therapy before the start of ICB therapy.

Antibiotics use is negatively associated with PFS and OS in patients with cancer treated with ICI (7). In this study, 46 of 118 patients (39%) received antibiotic therapy. We considered that comorbidities which required the use of antibiotics could be one of the confounding factors, and antibiotic use was used as one of the background factor for propensity score analysis, which confirmed that probiotic CBT compared with no probiotic CBT was independently associated with improved survival outcomes.

Clostridium butyricum increases *Bifidobacterium* and reduces intestinal epithelial damage (8, 13–15). Intriguingly, Sivan and colleagues, reported that commensal *Bifidobacterium* promotes dendritic cell function and T-cell–directed antitumor immunity leading to improved ICB efficacy in a tumor-bearing murine model (5). These

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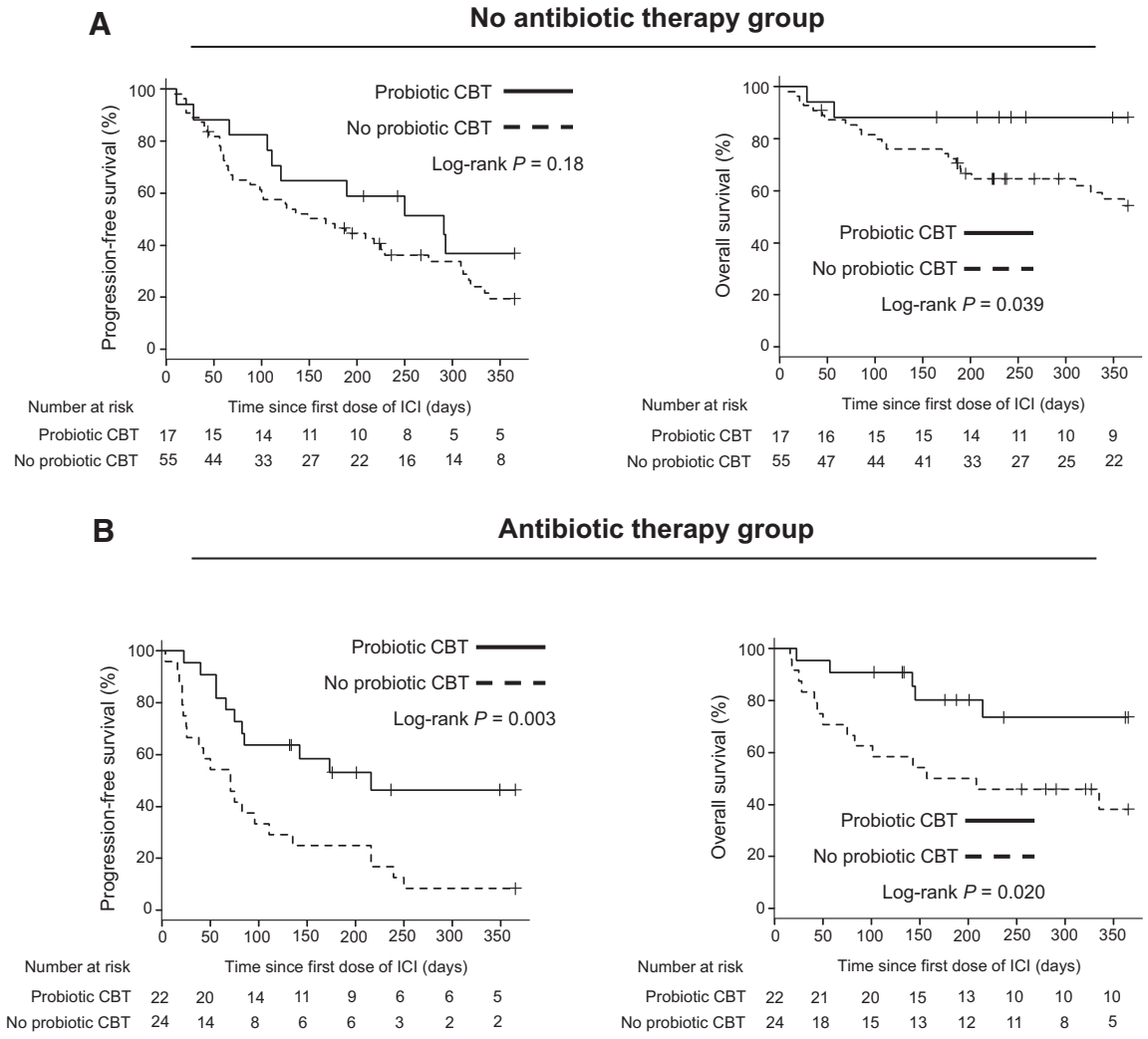

Figure 2.

Association between probiotic CBT and survival in patients who received antibiotic therapy within 60 days before the start of ICB therapy. **A,** PFS and OS in patients with NSCLC who did not receive antibiotic therapy within 60 days before the start of ICB therapy, stratified by administration of probiotic CBT is shown. **B,** PFS and OS in patients with NSCLC who received antibiotic therapy within 60 days before the start of ICB therapy, stratified by administration of probiotic CBT is shown.

data suggest that *Clostridium butyricum* may improve the efficacy of ICB through increasing commensal *Bifidobacterium*.

Gut microbiota modulate immune responses (4, 5, 8, 18). *Clostridium butyricum* reduce systemic Th17 cells in a murine model of inflammatory disease (8). Th17 cells promote invasion and migration of cancer cells, and cancer stem cell–like properties via STAT3 signaling in NSCLC, suggesting *Clostridium butyricum* may inhibit the aggressiveness through the reduction of Th17 cells in NSCLC treated with ICB (19).

In preclinical murine models of probiotic CBT, modulation of intestinal flora balance and gut metabolic alterations are observed 14 to 21 days after probiotic CBT administration (13, 14). However, the duration of the effects of probiotic CBT on systemic immunity has not been elucidated in patients with cancer who receive ICB therapy. Thus, we used 6 months prior to start of ICB as a cutoff for defining use of probiotic CBT before ICB initiation to maximally detect the impact of probiotic CBT on clinical outcomes of patients with lung cancer treated with ICB.

In subgroup analysis of patients with or without antibiotic therapy prior to initiation of ICB, the impact of probiotic CBT on patients' survival was more significant in patients with cancer who received antibiotic therapy than those who did not receive antibiotic therapy, suggesting that a clinical setting of probiotic CBT under the condition of antibiotic use may be necessary to maximize the benefit from probiotic CBT and detect a beneficial effect of probiotic CBT. Probiotic CBT improves dysbiosis under the condition of antibiotic therapies (8–11, 13–15). *Bifidobacterium*, which promotes antitumor immunity and facilitates efficacy of ICB, is significantly increased in combination with probiotic CBT and clindamycin when compared with only probiotic CBT (13, 15), suggesting that a combination therapy of probiotic CBT with antibiotics to deplete microbial communities could potentially improve responses to ICB therapy (20). We speculate that a beneficial impact on gut microbiome by probiotic CBT might have enhanced under the condition of antibiotic use and led to significant survival benefit coupled with the reduced patients' survival due to antibiotic-related gut dysbiosis in group not treated with

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probiotic CBT. However, our results from subgroup analysis should be interpreted with caution due to small sample sizes. Profiling of the gut microbiome in patients with cancer who received probiotic CBT with/without antibiotics during ICB therapy is essential to elucidate the mechanism of impact of probiotic CBT on clinical outcomes.

Human gut mucosal probiotic colonization is significantly enhanced by antibiotics (21), suggesting that gut mucosal colonization of probiotic *Clostridium butyricum* might be enhanced by antibiotics in patients with cancer. Postantibiotic gut mucosal microbiome reconstitution is impaired by probiotics (21). Dysbiosis of the gut microbiota is implicated in carcinogenesis and impaired response to cancer therapies, indicating an unfavorable microbiota already exists in patients with advanced cancer (20, 22, 23). These results suggest that probiotic CBT in combination with antibiotics may shift the preexisting gut dysbiosis in patients with cancer to a favorable microbiota and delay an unfavorable gut microbiome reconstitution. This hypothesis should be validated by correlative analyses of the gut microbiota in prospective studies.

In contrast to the results reported previously (1, 3, 4, 7), antibiotic therapy prior to initiation of ICB was not significantly associated with worse clinical outcomes with ICB therapy. We consider that this discrepancy in the results of impact of antibiotic therapy on clinical outcomes between our study and previous studies might have occurred because 22 of 46 patients (47%) had received probiotic CBT in antibiotic therapy group. In this study, we found that probiotic CBT was significantly associated with improved survival outcomes in the cohort of patients with NSCLC who received antibiotic therapy. These results support our hypothesis that probiotic CBT may improve the decreased efficacy of ICB in patients treated with antibiotics.

Because of the lack of objective criteria to evaluate whether probiotic CBT was effective or not effective, we could not assess the effectiveness of probiotic CBT in this retrospective study. The association of the efficacy of probiotic CBT for symptoms related to dysbiosis with survival benefits in patients with cancer treated with ICB need to be assessed in prospective studies.

Characteristics such as diet, lifestyle, or genetics can affect the composition of the gut microbiota. The ethnic origin of individuals is an important factor to be considered in microbiome research (24). In our study, only Japanese patients have been analyzed, which is a limitation.

Our study has another limitation. We speculate that *Clostridium butyricum* may modulate gut microbiota and shift an unfavorable antimicrobial-associated or nonantimicrobial-associated dysbiosis to a favorable microbiota, leading to an increase ICI clinical activity. However, we did not characterize the mechanism by which probiotic CBT exerts a positive effect on clinical outcomes. Profiling of the gut microbiome during ICI therapy with or without probiotic CBT is essential to elucidate the mechanism of impacts of probiotic CBT on clinical outcomes.

In conclusion, our findings support the hypothesis that probiotic CBT may have a positive impact on the therapeutic efficacy of ICB, providing rationale for combining probiotic therapy with immunotherapies. Despite being limited by the small sample size, retrospective study, heterogeneity of study cohort, and the lack of correlative analyses on patients' gut microbiota and impacts of probiotic CBT on systemic immunity, our findings provide the first evidence that manipulating commensal microbiota by probiotic CBT may enhance the efficacy of ICB and also suggest that probiotic CBT may improve the efficacy of ICB deteriorated by antibiotics in patients with lung cancer.

Disclosure of Potential Conflicts of Interest

T. Ikeda reports grants from JSPS KAKENHI (grant number 20K10323) outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Y. Tomita: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **T. Ikeda:** Data curation, software, formal analysis, validation, visualization, methodology, writing–original draft, writing–review and editing. **S. Sakata:** Resources, data curation, validation, investigation, methodology, writing–original draft. **K. Saruwatari:** Resources, data curation, validation, investigation, methodology. **R. Sato:** Conceptualization, resources, data curation, investigation, methodology, writing–original draft. **S. Iyama:** Resources, data curation, validation. **T. Jodai:** Resources, data curation, validation. **K. Akaike:** Resources, data curation, validation. **S. Ishizuka:** Resources, data curation, validation. **S. Saeki:** Resources, data curation, validation, writing–original draft. **T. Sakagami:** Supervision, validation, methodology, writing–original draft, writing–review and editing.

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