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ORIGINAL ARTICLE

Presence of few PD-1-expressing tumor-infiltrating immune cells is a potential predictor of improved response to salvage chemotherapy following nivolumab for non-small cell lung cancer: An exploratory case series

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Exhausted T cell; immunotherapy; PD-1; salvage chemotherapy.

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Abstract

Background: The combination of PD-1 inhibitors and cytotoxic drugs is reported to enhance anti-tumor activity in non-small cell lung cancer; however, the underlying synergistic mechanisms remain uncertain. This retrospective case series was designed to investigate objective response and survival rates of salvage chemotherapy following nivolumab and explore the immunohistochemical profiles of tumor-infiltrating immune cells.

Methods: The medical records of 37 patients administered nivolumab were retrospectively reviewed. Overall response rate and progression-free survival were compared among three groups: salvage chemotherapy following nivolumab, nivolumab therapy alone, and chemotherapy preceding nivolumab.

Results: Eight cases met the study criteria. Salvage chemotherapy following nivolumab improved the overall response rate to 62.5% (95% confidence interval [CI] 34.4–90.6%; $P = 0.004$) and median progression-free survival to six months (95% CI 4.6–7.4; $P = 0.016$), compared to nivolumab alone and preceding chemotherapy. The response to salvage chemotherapy was not associated with tumor PD-L1 expression. A partial response was achieved in four cases with ≤ 5% and ≤ 2.9 cells/mm^2 of PD-1$^+$ immune cells, whereas stable disease and progressive disease were observed in three cases with ≥ 30% and ≥ 12.7 cells/mm^2. Responders had fewer PD-1$^+$ immune cells than non-responders (percentage $P = 0.028$; density $P = 0.034$).

Conclusion: Salvage chemotherapy following nivolumab improved anti-tumor activity regardless of tumor PD-L1 status, but nivolumab following chemotherapy did not. The presence of few PD-1$^+$ tumor-infiltrating immune cells may serve as a potential predictor of response to salvage chemotherapy. Further studies involving a large cohort are needed to clarify how nivolumab re-sensitizes the tumor immune microenvironment to chemotherapy.

Introduction

PD-1 inhibitors, such as nivolumab, have emerged as breakthrough therapy for non-small cell lung cancer (NSCLC). We have reported the drastic regression of lung cancer cases treated with chemotherapy following nivolumab, which is known as salvage chemotherapy.[1] Several recent studies have also shown that the combination therapy of PD-1 inhibitors with anti-cancer drugs, including

salvage chemotherapy, produces high anti-tumor activity in some patients.[2–7] Salvage chemotherapy showed a high objective response rate of 38% (95% confidence interval [CI] 31.3–44.7%) in a total of 200 cases obtained from four retrospective studies.[2–5]

However, the mechanism that underlies this immunochemotherapy and whether optimal patients can be selected in advance are unknown. The evaluation of salvage chemotherapy may provide clues to these issues, because the anti-tumor effects of PD-1 inhibitors and chemotherapy are considered separately. This preliminary case series was designed to investigate the response rate and survival according to the sequence of chemotherapy and nivolumab, and to explore the tumor immune microenvironment using tissue samples taken at initial diagnosis.

Methods

Study design

This retrospective, single-institute, exploratory case series was conducted at Sasebo City General Hospital, which is officially authorized as a regional core cancer center. Patients were included in the present study based on the following two criteria: they had recurrent or advanced NSCLC, and underwent salvage chemotherapy following nivolumab between April 2016 and December 2017. Lung cancer stage was determined according to the 8th edition of the Union for International Cancer Control classification. The therapy regimens were decided by discussion between the physicians and patients. The patients received 3 mg/kg of nivolumab intravenously every two weeks. All patients were followed-up until 1 June 2018. Patients were excluded from the analysis if they had any other neoplasms requiring concurrent treatment, if their lung cancer harbored *EGFR* mutations or *ALK* rearrangements, and if they had received other immune checkpoint inhibitors.

The institutional review board of Sasebo City General Hospital approved the study protocol (approval number 2018-A012). Informed consent was obtained from the patients by opt-out on the hospital website, in accordance with the ethical guidelines presented by the Japanese Ministry of Health, Labor and Welfare.[8] This study was registered with the University Hospital Medical Information Network in Japan (registry number: UMIN000032667). Three cases in the study population were previously published as case reports.[1,9]

Data collection

The records of patients receiving nivolumab were extracted from the database of the institutional multidisciplinary medical team for immune checkpoint inhibitors. Demographics, histology, molecular profiling, imaging, and treatment history were reviewed. A systemic follow-up survey of the lesions was performed by physical examination, chest radiography, and blood tests, including serum tumor markers, at least once a month. Computed tomography scans of the chest and upper abdomen, brain magnetic resonance imaging, and positron emission tomography were routinely conducted as scheduled for outpatient follow-up. Two experts, blinded to the clinical data, separately assessed the best tumor responses according to Response Evaluation Criteria in Solid Tumors version 1.1. In cases of a discrepancy, the experts reached agreement by means of re-analysis and discussion. Treatment-related adverse events were assessed according to Common Terminology Criteria for Adverse Events version 4.0.

Immunohistochemistry

Immunohistochemical findings of formalin-fixed, paraffin-embedded tumor specimens taken at initial diagnosis, including PD-L1 expression on cancer cells, $CD8^+$ T cells, $PD\text{-}1^+$ immune cells, and $FOXP3^+$ regulatory T cells, were examined. The antibody clones used were as follows: PD-L1 (22C3, Dako, Santa Clara, CA, USA); CD8 (4B11, Leica Biosystems, Nussloch, Germany); PD-1 (SP269, Spring Bioscience, Pleasanton, CA, USA); and FOXP3 (236A/E7, Abcam, Cambridge, UK). Positivity of PD-L1 for tumor cells and of CD8, PD-1, and FOXP3 for tumor-infiltrating immune cells was expressed as a percentage of positive cells in the four representative areas. Additionally, the number of $PD\text{-}1^+$ immune cells was expressed as the density of positive cells in the four representative areas. Two independent experts blinded to the clinical data evaluated the immunohistochemical findings. If the assessments differed, the experts re-evaluated the specimen and reached a consensus.

Statistical analyses

The overall response rate (ORR) was expressed with 95% confidence interval (CI), and differences between groups were statistically evaluated by Fisher's exact test. Progression-free survival (PFS) from the initiation of each therapy was estimated using the Kaplan–Meier method, with differences analyzed by the log-rank test. Responders to salvage chemotherapy were defined as those with a complete response (CR) or partial response (PR), while non-responders were those with stable disease (SD) or progressive disease (PD). The differences in immunohistochemical findings between the responders and non-responders were assessed using the Mann–Whitney *U* test. Data were analyzed using the SAS software program (SAS Institute Inc.,

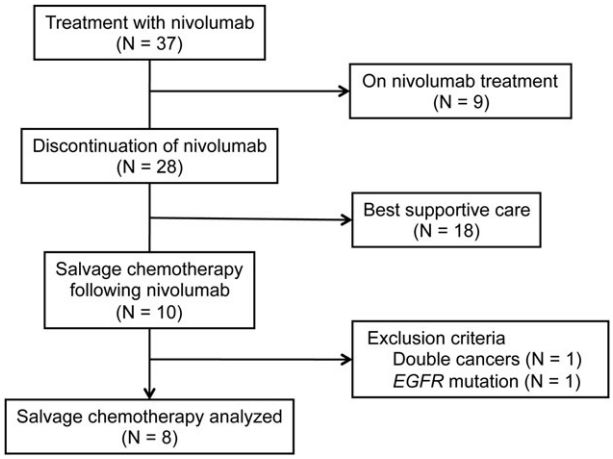

Figure 1 Study flow diagram.

Cary, NC, USA), and a two-tailed P value < 0.05 was considered significant.

Results

Patient characteristics

A total of 37 patients received nivolumab, including three who achieved CR and five PR as their best response (Fig 1). The ORR of nivolumab therapy in the 37 patients was 21.6% (95% CI 8.4–34.8%). As of June 2018, 9 patients were still being treated with nivolumab, and 18 did not receive salvage chemotherapy but best supportive care as a result of disease exacerbation or adverse events. Of the remaining 10 patients, 2 were excluded because of the presence of concurrent uterine cancer or *EGFR* mutation. Thus, the data of eight patients were analyzed in the present study. The patients' characteristics are summarized in Table 1.

All patients received platinum doublet therapy as first-line chemotherapy. The median number of nivolumab administrations was 7, and the median period between the last nivolumab and first salvage chemotherapy administration was 21 days. Three patients underwent salvage chemotherapy as third-line therapy, and five as fourth-line or higher (nivolumab was included as a line of therapy). Five patients received single-agent S-1 and three received combination therapy including taxanes as salvage chemotherapy. Two patients developed grade 3 anemia after exposure to S-1 and grade 3 pneumonitis after treatment with docetaxel/ramucirumab.

Changes in overall response rate (ORR) and progression-free survival (PFS) by consecutive therapy

Overall response rate and PFS were compared among three groups: salvage chemotherapy following nivolumab, nivolumab therapy alone, and chemotherapy preceding nivolumab. As shown in Table 2, the ORR after salvage chemotherapy was 62.5% (5/8, 95% CI 34.4–90.6%), whereas the ORRs of nivolumab therapy alone or preceding chemotherapy were both 0% (0/8, 95% CI 0–21.0%). The ORR after salvage chemotherapy was significantly higher than in the other groups (P = 0.004). The median PFS after salvage chemotherapy was 6 months (95% CI 4.6–7.4) compared to 3.5 (95% CI 2.0–5.0) and 3.6 (95% CI 2.6–4.7) months after nivolumab therapy and preceding chemotherapy, respectively (Fig 2). PFS was significantly longer after salvage chemotherapy (P = 0.016).

Table 1 Patient characteristics (n = 8)

Variable	Number
Age, years	
Median (range)	69 (62–75)
Gender	
Male	6 (75%)
Female	2 (25%)
ECOG performance status	
0	1 (12.5%)
1	7 (87.5%)
Smoking status	
Former smoker	8 (100%)
Never-smoker	0 (0%)
Histologic subtype	
Adenocarcinoma	2 (25%)
Squamous cell carcinoma	4 (50%)
Undifferentiated carcinoma	2 (25%)
Stage	
III	1 (12.5%)
IV	5 (62.5%)
Recurrence	2 (25%)
Regimen of preceding chemotherapy before nivolumab	
Platinum doublet	5 (62.5%)
S-1	2 (25%)
Docetaxel	1 (12.5%)
Number of nivolumab administrations	
Median (range)	7 (2–10)
Period between the last nivolumab and first salvage chemotherapy, days	
Median (range)	21 (20–69)
Line of salvage chemotherapy	
Third-line	3 (37.5%)
Fourth-line	3 (37.5%)
Fifth-line	2 (25%)
Regimen of salvage chemotherapy	
S-1	5 (62.5%)
Carboplatin + albumin-bound paclitaxel	2 (25%)
Docetaxel + ramucirumab	1 (12.5%)

ECOG, Eastern Cooperative Oncology Group.

Table 2 Relationship between response to salvage chemotherapy and immunologic profiles of lung cancer

Case	Preceding chemotherapy		Nivolumab	Salvage chemotherapy		Histologic	PD-L1$^+$	CD8$^+$	PD-1$^+$ immune cells		FOXP3$^+$
	Regimen	Response	Response	Regimen	Response	subtype	tumor cells	T cells	%	/mm^2	Tregs
Case 1	DTX	SD	PD	S-1	PR	UN	95%	60%	< 1%	1.3	30%
Case 2	S-1	PD	SD	CBDCA/nab-PTX	PR	SCC	10%	40%	5%	2.9	5%
Case 3	CDDP/PEM/BEV	SD	PD	S-1	PR	AD	< 1%	30%	< 1%	1.5	5%
Case 4	CDDP/GEM	PD	PD	DTX/RAM	PR	SCC	< 1%	50%	< 1%	0.6	20%
Case 5	S-1	PD	PD	CBDCA/nab-PTX	PR	AD	NA	NA	NA	NA	NA
Case 6	CBDCA/nab-PTX	SD	PD	S-1	SD	SCC	20%	50%	30%	12.7	15%
Case 7	CBDCA/PTX	PD	PD	S-1	SD	UN	< 1%	60%	50%	24	20%
Case 8	NDP/DTX	PD	PD	S-1	PD	SCC	70%	60%	60%	33.9	15%

AD, adenocarcinoma; BEV, bevacizumab; CBDCA, carboplatin; CDDP, cisplatin; DTX, docetaxel; GEM, gemcitabine; NA; not available; nab-PTX, albumin-bound PTX; NDP, nedaplatin; PD, progressive disease; PEM, pemetrexed; PR, partial response; PTX, paclitaxel; RAM, ramucirumab; SCC, squamous cell carcinoma; SD, stable disease; Tregs, regulatory T cells; UN, undifferentiated carcinoma.

Tumor immune microenvironment according to response to salvage chemotherapy

The relationship between objective response to salvage chemotherapy and the profiles of the tumor immune microenvironment was explored (Table 2, Fig 3). One of eight patients was excluded from immunohistochemical analysis because of an inadequate tissue specimen. The objective response to salvage chemotherapy was not associated with the level of tumor PD-L1 expression or the degree of tumor-infiltrating CD8$^+$ T and FOXP3$^+$ regulatory T cells. PD-1$^+$ immune cells were mainly composed of lymphocytes, and the positivity of PD-1 on immune cells ranged from < 1% to 60%. Four responders had ≤ 5% of PD-1$^+$ immune cells, while three non-responders had ≥ 30% of PD-1$^+$ immune cells. Responders had a lower percentage of PD-1$^+$ immune cells than non-responders (P = 0.028). The number of PD-1+ immune cells ranged from 0.6 to 33.9 cells/mm^2. Responders had ≤ 2.9 cells/mm^2 PD-1$^+$ immune cells, while non-responders had ≥ 12.7 cells/mm^2 PD-1$^+$ cells. Responders had less PD-1$^+$ immune cells than non-responders (P = 0.034).

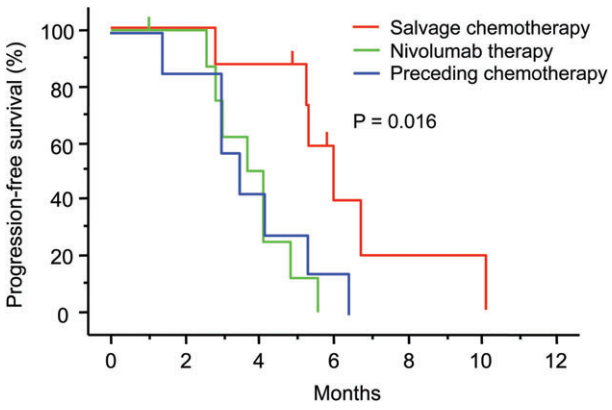

Figure 2 Kaplan–Meier estimates of progression-free survival according to consecutive therapies. The vertical bars indicate censored cases. Progression-free survival after salvage chemotherapy, nivolumab alone, and preceding chemotherapy (P = 0.016 by log-rank test).

Discussion

This exploratory case series provides two important conclusions. Salvage chemotherapy following nivolumab revived anti-tumor activity regardless of tumor PD-L1 expression, but nivolumab following chemotherapy did not. The presence of few PD-1$^+$ immune cells in the tumor may be a candidate therapeutic predictor of response to salvage chemotherapy.

The sequence of chemotherapy following nivolumab improved anti-tumor activity regardless of tumor PD-L1 expression, but the reverse sequence did not. Similar to these findings, four retrospective studies of salvage chemotherapy, including a case–control study, showed a high ORR of 38% and median PFS of approximately four months in previously treated patients with NSCLC.[2–5] In the case–control study, the odds ratio for achieving an objective response to salvage chemotherapy was more than three times higher.[2] However, previous clinical studies did not fully identify whether the anti-tumor activity of salvage chemotherapy was associated with the sequence of PD-1 inhibitors.

The sequence and timing of chemotherapy for PD-1 blockade are critical issues. Although chemotherapeutic agents have immunomodulatory effects,[10,11] subset analysis of a phase III trial of first-line pembrolizumab showed that lung cancer patients who received second-line chemotherapy following pembrolizumab had superior PFS compared to those who received second-line PD-1 inhibitors following chemotherapy.[12] In a preclinical model with melanoma, PD-1 blockade followed three days later with chemotherapy regressed the tumor and prolonged survival compared

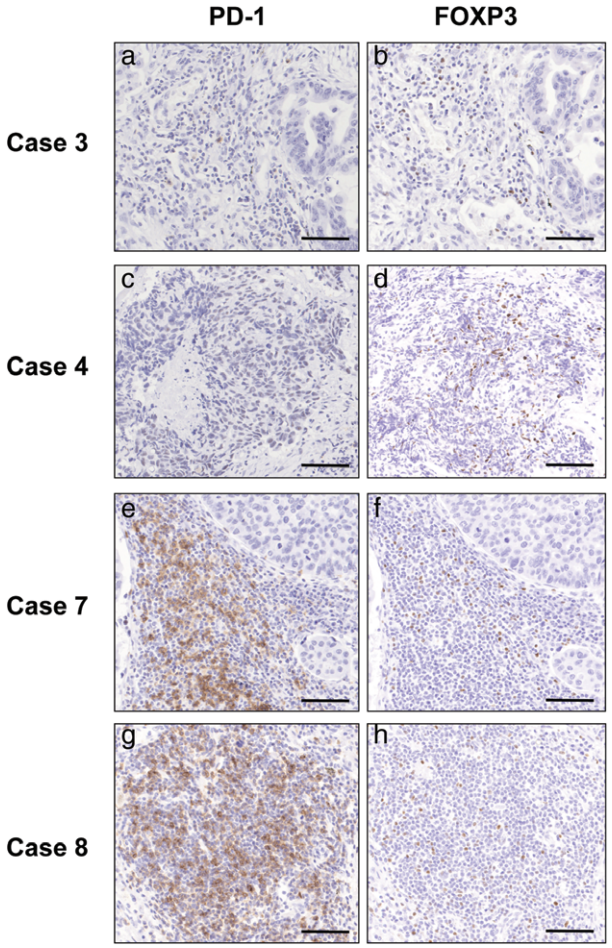

Figure 3 Immunohistochemical examination of the tumor-infiltrating immune cells in four representative cases. Case 3 of adenocarcinoma and case 4 of squamous-cell carcinoma show a partial response to salvage chemotherapy, case 7 of undifferentiated carcinoma shows stable disease, and case 8 of squamous-cell carcinoma shows progressive disease. (**a**,**c**,**e**,**g**) PD-1$^+$ immune cells; (**b**,**d**,**f**,**h**) FOXP3$^+$ regulatory T cells (scale bars 100 μm).

to chemotherapy alone, PD-1 blockade alone, and concurrent administration of these agents.[13] Although tumor growth in PD-1 knockout mice was similar to that of wild type mice, chemotherapy significantly suppressed tumor growth in PD-1 knockout mice.[13] Collectively, these findings (including ours) suggest that preceding nivolumab could synergize with salvage chemotherapy, even without tumor PD-L1 expression or an evident objective response to nivolumab.

The presence of few PD-1$^+$ tumor-infiltrating immune cells may be a candidate therapeutic predictor of salvage chemotherapy following nivolumab. PD-1-expressing CD8$^+$ cells consist of different populations that respond differentially to PD-1 blockade. PD-1 inhibitors can activate partially exhausted cytotoxic T cells and shrink the tumors, but not deeply exhausted T cells.[14] High PD-1-expressing CD8$^+$ cells secret less cytotoxic cytokines in vitro,[15,16] but nivolumab does not subsequently restore the release of cytokines.[17] Although a subset of effector T cells differentiated into effector memory T cells for durable effects of PD-1 inhibitors,[13,14] the deeply exhausted T cells were not able to differentiate into effector memory T cells.[18] In a retrospective cohort study of lung cancer, a low ratio of PD-1$^+$ cells to CD8$^+$ cells in the tumor, but not the actual number of PD-1$^+$ cells, conferred better ORR and PFS after nivolumab therapy.[19] In the present study, a low ratio of PD-1$^+$ cells to immune cells and a small number of PD-1$^+$ cells were not associated with ORR after nivolumab therapy, but conferred better ORR after salvage chemotherapy. Although the reason for this discrepancy is unclear, recent studies have raised a fundamental question concerning the conflicting roles of PD-1 on T cells: can PD-1 expression be considered a reflection of tumor-specific T cell activation as well as T cell dysfunction in the dynamic process of the tumor immune microenvironment?[20] High PD-1-expressing CD8$^+$ cells may be deeply exhausted and irreversibly dysfunctional to PD-1 blockade followed by salvage chemotherapy.

Another important finding of our study was the coexistence of FOXP3$^+$ Tregs with CD8$^+$ T cells within the tumor. We reported that a case of lung cancer regressed spontaneously and subsequently relapsed with an increase in the number of Tregs in the circulation.[21,22] We also reported that lung cancer cases showing a remarkable response to nivolumab contained < 5% Tregs in the tumor.[23,24] Tregs inhibited nivolumab-stimulated release of interferon-γ from effector T cells in vitro.[25] Although PD-1 blockade did not affect the function of Tregs,[26] several anti-cancer drugs are known to reduce the number of Tregs and restore the activity of effector T cells.[27] In addition, the selective depletion of Tregs by the chemokine receptor 4 antagonist mogamulizumab has been suggested to revive sensitivity to chemotherapy.[28] These findings encourage further investigation to define whether Tregs may affect, at least in part, the efficacy of nivolumab and subsequent chemotherapy.

Several limitations of this study need to be acknowledged. First, most of the study cases received late-line chemotherapy, suggesting that they may have had indolent tumors or better clinical conditions. In order to reduce the effect of selection bias, we used a self-controlled case series method: the individuals acted as their own control, and the efficacy of salvage chemotherapy was compared to that of previous therapies within the individuals. Second, the sample cases incidentally consisted of patients without an objective response to nivolumab. This is mainly because the responders to nivolumab remain on the treatment. It is poorly documented whether the underlying mechanisms of

the innate primary resistance to nivolumab differ from those of acquired resistance.[14] Nevertheless, a retrospective cohort study suggests that the response to salvage chemotherapy seems similar in the presence or absence of the response to PD-1 blockade.[5]

The third limitation is that the tumor immune microenvironment was evaluated using tissue specimens taken at initial diagnosis. In clinical practice, re-biopsy of tumor tissue for salvage chemotherapy is difficult. However, it is well known that immunotherapy can alter the characteristics of the tumor immune microenvironment.[29] Additionally, monitoring the phenotype and frequency of blood-circulating immune cells may be useful to clarify the mechanisms underlying immunotherapy and salvage chemotherapy.[30] In patients with melanoma receiving salvage chemotherapy, the preexistence or increase of blood-circulating $CX3CR1^+$ $CD8^+$ effector memory T cells, which frequently expressed PD-1, was associated with anti-tumor activity.[13] The present preliminary findings warrant prospective clinical trials in a large cohort undergoing re-biopsy of tumor tissue or monitoring of blood-circulating immune cells before and during salvage chemotherapy.

In conclusion, to the best of our knowledge, this article is the first to report that the sequence of chemotherapy following nivolumab could enhance anti-tumor activity regardless of tumor PD-L1 expression, but not the reverse sequence. Furthermore, the presence of few $PD\text{-}1^+$ tumor-infiltrating immune cells may serve as a potential predictor of response to salvage chemotherapy. These findings may provide insight into a better approach for immune escape in lung cancer. Further studies are required to elucidate how nivolumab renders the tumor immune microenvironment more susceptible to chemotherapy specifically.

Disclosure

No authors report any conflicts of interest.

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