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The relationship between galectin-1 expression and the efficacy of anti-PD-1 therapy for non-small cell lung cancer patients

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We herein examined the potential of galectin-1 expression to predict the efficacy of anti-PD-1 therapy for patients with non-small cell lung cancer (NSCLC). We also investigated the potential of galectin-1-targeted therapy as an immunotherapeutic approach for patients with NSCLC. This retrospective cohort study included patients with metastatic, unresectable, or postoperative recurrent NSCLC who were treated with anti-PD-1 monotherapy (nivolumab or pembrolizumab) between November 2017 and August 2019. Galectin-1 expression in the plasma and tumor tissues of NSCLC patients before the initiation of anti-PD-1 therapy was analyzed using ELISA and immunohistochemistry. To examine the relationship between galectin-1 expression and anti-tumor T-cell activity, the cytotoxicity of peripheral human T cells against tumor cells was assessed using bispecific T-cell engager (BiTE) technology (BiTE assay). Plasma galectin-1 concentrations were significantly higher in NSCLC patients before the initiation of anti-PD-1 therapy than in healthy donors. Median progression-free survival with anti-PD-1 therapy was slightly extended in the group with low galectin-1 expression in both plasma and tumor tissue, although the difference was not statistically significant. T-cell cytotoxicity in the BiTE assay was stronger against galectin-1 knockout tumor cells than wild-type tumor cells. In conclusion, a relationship is suggested between galectin-1 expression and the efficacy of anti-PD-1 therapy in NSCLC patients.

Keywords Non-small cell lung cancer, Galectin-1, PD-1, T cells, Immunotherapy

Immune checkpoint inhibitors (ICIs), including anti-programmed death-1 (PD-1) therapy, benefit a limited population of cancer patients. Therefore, predictive biomarkers for ICIs are needed for personalized medicine for cancer patients. Several biomarkers are utilized for ICIs. Programmed death ligand-1 (PD-L1) staining was adapted to companion diagnostics for anti-PD-1 therapy[1,2]. PD-L1 on tumor cells is the direct target for anti-PD-1 therapy[3]. Microsatellite instability (MSI) and tumor mutational burden (TMB) are also utilized as predictive biomarkers for anti-PD-1 therapy[4–7]. MSI and TMB are related to the expression of neoantigens on tumor cells, which is the direct target of cytotoxic T lymphocytes[8,9]. These biomarkers are involved in the mechanisms of action of ICIs. Besides PD-1, several immune checkpoint molecules are related to T-cell immunity. Lymphocyte Activation Gene-3 (LAG-3) is one of the suppressive immune checkpoint molecules expressed in T cells[10]. The therapeutic efficacy of anti-PD-1 therapy was lower in cancer patients with the high expression of LAG-3 on T cells than in those with the low expression of LAG-3 [11,12]. The combination of relatlimab, a LAG-3-inhibiting

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Variables	All ($N=40$)	Plasma galectin-1 concentration high ($N=17$)	Plasma galectin-1 concentration low ($N=23$)	P value
Age, years	70.5 (40–82)	70 (40–82)	71 (40–82)	0.79**
Male	27 (67.5%)	14 (82.4%)	13 (56.5%)	0.10*
Smoker	36 (90%)	16 (94.1%)	20 (87.0%)	0.62*
Histology Squamous	11 (27.5%)	5 (29.4%)	6 (26.1%)	1.0*
PD-L1 expression level ≥50%	28 (70%)	11 (64.7%)	16 (69.6%)	0.73*
Anti-PD-1 therapy 1st line	23 (57.5%)	7 (41.2%)	16 (69.6%)	0.11*

Table 1. Baseline characteristics of NSCLC patients with plasma galectin-1 concentrations measured at the initiation of anti-PD-1 therapy. * Fisher's exact test ** The Student's t-test Data are presented as medians (range) or N (%). NSCLC, non-small cell lung cancer; PD-1, programmed death 1; PD-L1, rogrammed death ligand 1.

Variables	Healthy donors ($N=10$)
Age, years	34.5 (32–56)
Male	6 (60.0%)

Table 2. Baseline characteristics of healthy donors. Data are presented as medians (range) or N (%).

antibody, and nivolumab, a PD-1-inhibiting antibody, achieved longer progression-free survival (PFS) than nivolumab monotherapy in patients with previously untreated metastatic or unresectable melanoma[13]. The T-cell immunoreceptor with immunoglobulin and tyrosine-based inhibitory motif domain is another suppressive immune checkpoint molecule that is also a predictive biomarker for anti-PD-1 therapy[14,15]. These immune checkpoint molecules are potential targets of combinational immunotherapy with PD-1 blockade[13]. The identification of promising immune checkpoint molecules will lead to the development of more biomarkers and therapeutics for cancer immunotherapy.

Galectin-1 has been shown to suppress T-cell activity by inducing T-cell apoptosis and suppressing T-cell receptor (TCR) signal transduction[16,17]. When secreted from tumor cells, galectin-1 binds to the surface of tumor cells and inhibits T-cell activity[18]. Therefore, galectin-1 is one of the immune checkpoint molecules for T-cell immunity. We previously reported that tetracyclines enhanced anti-tumor T cell activity and also that galectin-1 was a target molecule for tetracycline in this mechanism of action[19–22]. Several galectin-1 inhibitors are currently being investigated in clinical trials[23]. However, the clinical relevance of targeting galectin-1 for immunotherapy for non-small cell lung cancer (NSCLC) remains unclear.

In the present study, we examined the potential of galectin-1 expression to predict the efficacy of anti-PD-1 therapy for NSCLC patients. We also investigated the effects of galectin-1-targeted treatment in combination with anti-PD-1 therapy in in vitro experiments using the T cells of NSCLC patients.

Results

Correlation of plasma galectin-1 concentrations with the treatment efficacy of anti-PD-1 therapy in NSCLC patients

Forty patients treated with nivolumab or pembrolizumab for NSCLC were enrolled in the present study (Table 1). The median follow-up time after anti-PD-1 therapy was 540 days (range, 40-1310 days). Peripheral blood was collected from all 40 patients before the initiation of anti-PD-1 therapy. NSCLC biopsy specimens collected by bronchoscopy, computed tomographic-guided needle biopsy, or surgery before the initiation of anti-PD-1 therapy were available from 32 patients.

Galectin-1 concentrations in plasma isolated from the peripheral blood of NSCLC patients ($N=40$) and healthy donors ($N=10$, Table 2) were measured by ELISA. Median plasma galectin-1 concentrations were higher in NSCLC patients before the initiation of anti-PD-1 therapy than in healthy donors (14.87 ng/mL vs. 7.45 ng/mL, $p=0.0030$) (Fig. 1A). Plasma galectin-1 concentrations were slightly higher in NSCLC patients whose treatment response to anti-PD-1 therapy was progressive disease (PD) ($N=8$) than in those whose treatment response was stable disease (SD) and a partial response (PR) ($N=28$) (four NSCLC patients had no target lesion that was assessable based on RECIST 1.1) (Fig. 1B). To examine the potential of plasma galectin-1 concentrations to predict responses to anti-PD-1 therapy, the area under the receiver operating characteristic curve (AUC) for plasma galectin-1 concentrations was examined. The AUC was 0.67 for differentiating PR and SD from PD for the efficacy of anti-PD-1 therapy with a cut-off value of 15.29 ng/mL (sensitivity = 64.3%, specificity = 75.0%, $p=0.15$) (Fig. 1C).

Based on the cut-off value of 15.29 ng/mL for plasma galectin-1 concentrations, patients with plasma galectin-1 concentrations higher than 15.29 ng/mL were grouped into the 'high group', while the remaining patients were grouped into the 'low group'. The baseline characteristics of the two groups at the initiation of anti-PD-1 therapy are summarized in Table 1. No significant differences were observed in age, tissue type, PD-L1 expression level, or anti-PD-1 therapy treatment line. Median PFS with anti-PD-1 therapy was slightly longer in the low group than in the high group; 12.7 (95% confidence interval [CI] 4.6–21.3) months vs. 5.1 (95% CI

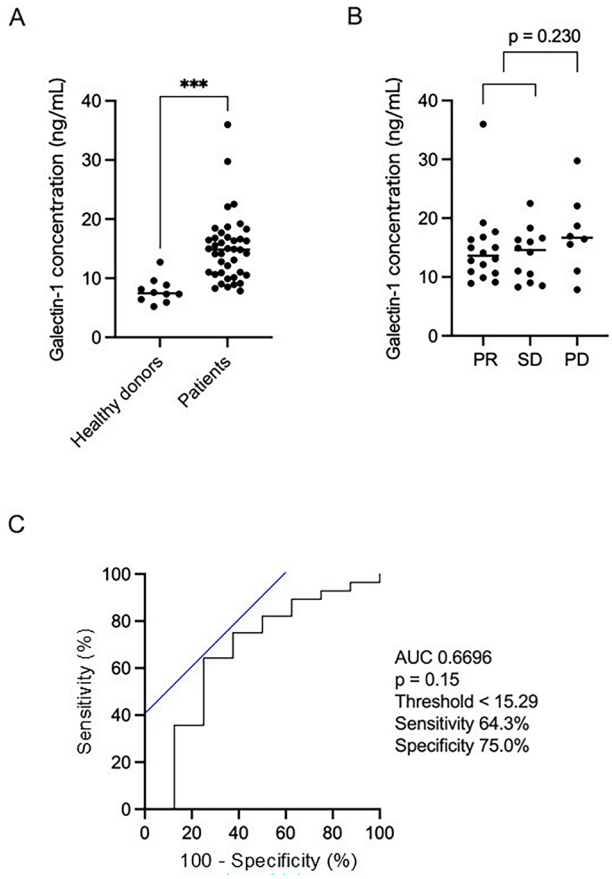

Fig. 1. Relationship between plasma galectin-1 concentrations and the treatment efficacy of anti-PD-1 therapy in NSCLC patients. (**A**) Galectin-1 concentrations in the plasma of NSCLC patients ($N = 40$) and healthy donors ($N = 10$) were measured by ELISA. Galectin-1 concentrations in NSCLC patients were measured before the initiation of anti-PD-1 therapy. (**B**) Plasma galectin-1 concentrations were evaluated by the treatment efficacy of anti-PD-1 therapy in NSCLC patients (PR, $N = 16$; SD, $N = 12$; PD, $N = 8$). (**C**) ROC curves for plasma galectin-1 concentrations to differentiate NSCLC patients with PR and SD from those with PD. Data are shown as the mean ± SEM. An unpaired two-tailed Student's *t*-test was used to examine the significance of differences between samples (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant). PD-1, programmed death 1; ELISA, enzyme-linked immunoassay; PR, partial response; SD, stable disease; PD, progressive disease; ROC, receiver operating characteristic.

1.1–9.9) months, $p = 0.065$ (Fig. 2). Similarly, median OS with anti-PD-1 therapy was slightly longer in the low group than in the high group; 23.8 months vs. 19.0 months (Fig. 2).

These results suggested a potential association between galectin-1 expression and the efficacy of anti-PD-1 therapy in NSCLC patients, although the correlation did not reach statistical significance. Clinically, immunosuppressive mechanisms beyond galectin-1, such as the expression of alternative immune checkpoint molecules, could also impact the therapeutic efficacy of anti-PD-1 treatment.

Relationship between plasma galectin-1 expression on tumor cells and the treatment efficacy of anti-PD-1 therapy in NSCLC patients

To evaluate the prognostic significance of galectin-1 expression on tumor cells for anti-PD-1 therapy, we investigated galectin-1 expression in the NSCLC tissues of biopsy specimens by immunohistochemical staining. The immunohistochemical staining of NSCLC biopsy specimens was followed by an evaluation of the intensity of galectin-1 expression on the tumor cell surface by a pathologist (Table 3; Fig. 3A, Supplementary Fig. 1). We classified the intensity of galectin-1 expression on tumor cells into 5 categories [none ($N = 2$), 0%; weak ($N = 19$), > 0% and ≤ 1%; moderate ($N = 5$), > 1% and ≤ 10%; strong ($N = 2$), > 10% and ≤ 50%; very strong ($N = 3$), > 50%]. The immunohistochemical intensity of galectin-1 expression was classified into 5 categories. (Supplementary Fig. 2). For simplicity, patients evaluated as none or weak were classified into the 'negative group' ($N = 21$), while those evaluated as moderate, strong, or very strong were classified into the 'positive group' ($N = 10$). Plasma galectin-1 concentrations were modestly reduced in the negative group, although the difference did not reach statistical significance (Fig. 3B). Median PFS with anti-PD-1 therapy was slightly extended in the negative group, although the difference was not statistically significant [PFS 12.8 (95% CI 2.7–20.7) months vs. 3.3 (95% CI 0.49–9.9) months, $p = 0.208$]. No significant difference was observed in median OS with anti-PD-1 therapy

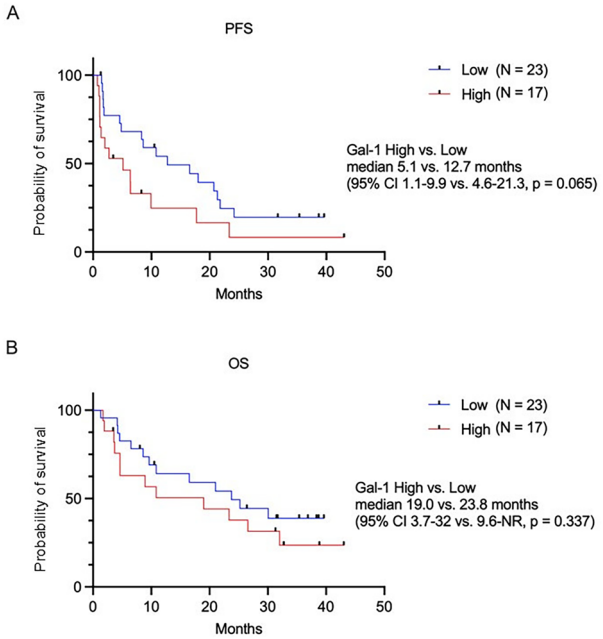

Fig. 2. Kaplan–Meier curves of (**A**) PFS and (**B**) OS with anti-PD-1 therapy in NSCLC patients whose plasma galectin-1 concentrations were low ($N = 23$) and high ($N = 17$). Overall survival curves were generated by the Kaplan–Meier method and compared using the Log-rank test. PFS, progression-free-survival; OS, overall survival; PD-1, programmed death 1; NR, not reached.

Percentage of galectin-1-positive tumor cells	Staining intensity	
0%	None	Negative
> 0% and ≤1%	Weak	
> 1% and ≤10%	Moderate	Positive
> 10% and ≤50%	Strong	
> 50%	Very strong	

Table 3. Immunohistological staining intensity of galectin-1 expression in tumor cells of lung cancer.

between the negative and positive groups [OS 23.4 (95% CI 4.2–NR) months vs. 18.7 (95% CI 0.59-NR) months, $p = 0.963$] (Fig. 3C).

Similar to plasma galectin-1 concentrations, a potential association is suggested between galectin-1 expressed on tumor cells and the treatment efficacy of anti-PD-1 therapy in NSCLC patients.

Galectin-1 expression was associated with anti-PD-1 therapy in vitro

Based on the negative effects of galectin-1 expression on the efficacy of anti-PD-1 therapy in NSCLC patients, we conducted in vitro experiments on the mechanisms underlying the inhibitory effects of galectin-1 on anti-PD-1 therapy. We previously reported that in vitro peripheral T-cell cytotoxicity assessed using BiTE (BiTE assay) correlated with both intratumoral T-cell activity and the treatment efficacy of anti-PD-1 therapy[24,25]. Using the BiTE assay, we herein investigated the relationship between plasma galectin-1 concentrations and peripheral T-cell cytotoxicity in NSCLC patients. When NSCLC patients were divided into high and low groups based on peripheral T-cell cytotoxicity, as described in our previous study (cut-off value of 17%) [24], plasma galectin-1 concentrations were higher in the low group than in the high group (Fig. 4A).

We then examined the relationship between galectin-1 expression on tumor cells and peripheral T-cell cytotoxicity using galectin-1 WT and KO tumor cells. Peripheral T-cell cytotoxicity in NSCLC patients was higher against galectin-1 KO tumor cells than WT tumor cells (Fig. 4B). Although galectin-1 was not detected in the cell culture supernatant of the co-culture with galectin-1 KO tumor cells and healthy donor PBMCs in BiTE assays with nivolumab, galectin-1 concentrations increased under the condition with nivolumab in BiTE assays against WT tumor cells (Fig. 4C, Supplementary Fig. 3). Galectin-1 secretion was enhanced more with activated NSCLC PBMCs by BiTE and WT tumor cells than by WT tumor cells alone without co-culture conditions (Supplementary Fig. 4), suggesting that the galectin-1 concentrations of NSCLC patients (patient numbers 1, 2, and 3) reached the upper limit of the co-culture conditions. These results show that galectin-1 expression was associated with anti-tumor T-cell activity and anti-PD-1 therapy.

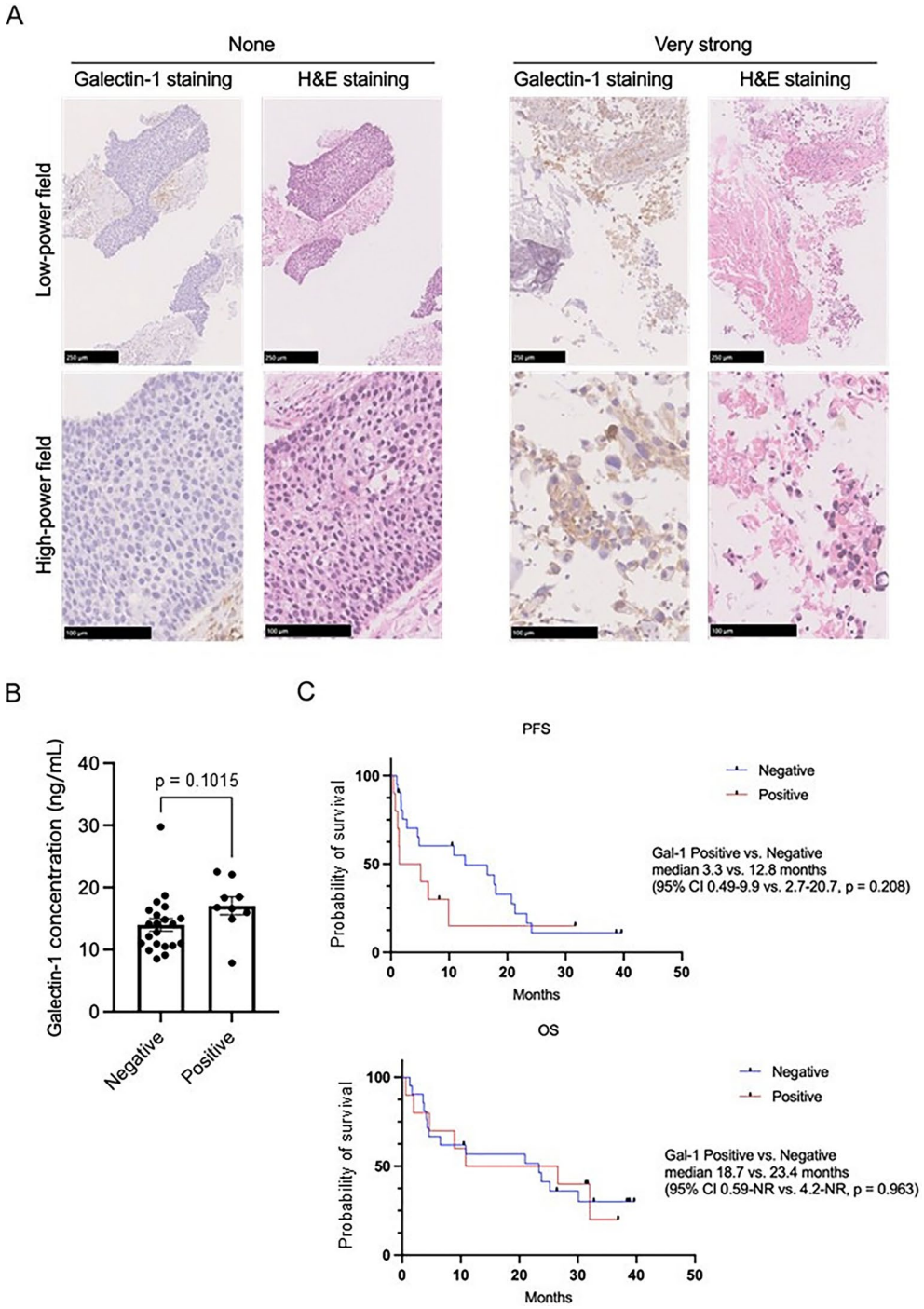

Fig. 3. Relationship between immunohistochemical galectin-1 expression on tumor cells and the treatment efficacy of anti-PD-1 therapy in NSCLC patients. (**A**) The immunohistochemical staining intensity of galectin-1 expression on tumor cells of NSCLC was measured. Representative images of immunohistochemistry assays with galectin-1 staining and hematoxylin and eosin (H&E) staining are shown. The left panel shows a case grouped into 'None', while the right panel shows a case grouped into 'Very strong'. (**B**) Relationship between the immunohistochemical staining intensity of galectin-1 expression on tumor cells (Negative, $N = 21$; Positive, $N = 9$) and plasma galectin-1 concentrations. (**C**) Kaplan–Meier curves of PFS and OS with anti-PD-1 therapy in NSCLC patients who were immunohistochemically negative ($N = 21$) and positive ($N = 10$) for galectin-1. Data are shown as the mean ± SEM. An unpaired two-tailed Student's t-test was used to examine the significance of differences between samples (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant). Overall survival curves were generated by the Kaplan–Meier method and compared using the Log-rank test. PD-1, programmed death 1; PFS, progression-free survival; OS, overall survival.

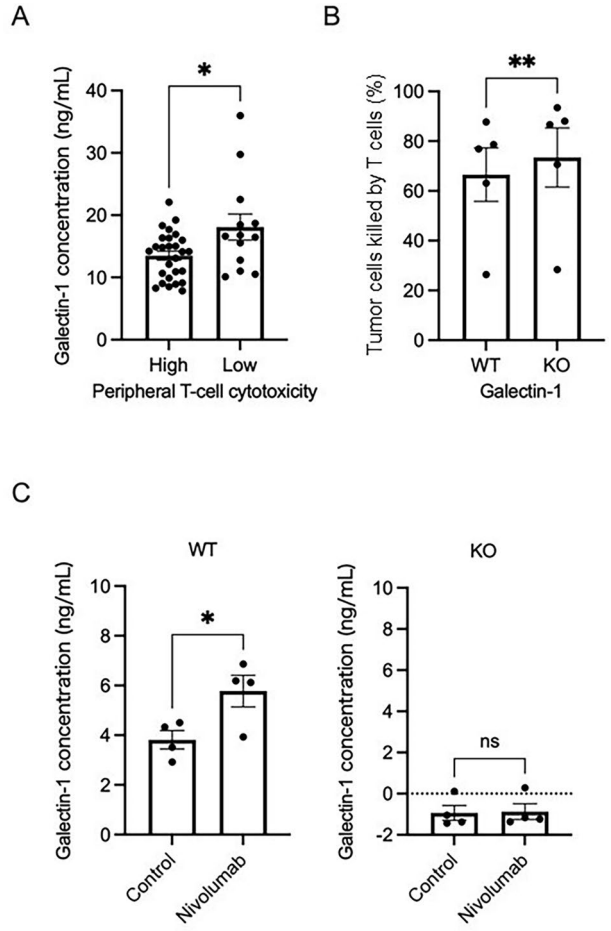

Fig. 4. In vitro validation assays of the relationship between galectin-1 expression and T-cell cytotoxicity. (**A**) Relationship between plasma galectin-1 concentrations and peripheral T-cell cytotoxicity in NSCLC patients measured by BiTE assays (High, $N=27$; Low, $N=13$). (**B**) Peripheral T-cell cytotoxicity in NSCLC patients was assessed using BiTE assays against WT or galectin-1 KO tumor cells ($N=5$). (**C**) Galectin-1 concentrations in cell culture supernatants were measured under conditions with and without nivolumab in BiTE assays against WT or galectin-1 KO tumor cells ($N=4$). The PBMCs of healthy donors were used in the assay. Data are shown as the mean ± SEM. An unpaired or paired two-tailed Student's *t*-test was used to examine the significance of differences between samples (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; ns, not significant). NSCLC, non-small cell lung cancer; KO, knockout; WT, wild-type; PBMC, peripheral blood monocyte.

Collectively, these results demonstrate that galectin-1 expression correlated with the treatment efficacy of anti-PD-1 therapy in NSCLC patients. The results of in vitro experiments suggest that galectin-1 was associated with resistance to anti-PD-1 therapy.

Discussion

In the present study, a relationship is suggested between galectin-1 expression and the efficacy of anti-PD-1 therapy for NSCLC patients. Furthermore, galectin-1 expression was associated with resistance to anti-PD-1 therapy, suggesting the potential of galectin-1-targeted treatment in combination with anti-PD-1 therapy.

Previous studies examined resistance to anti-PD-1 therapy[26,27], and suggested that the underlying mechanisms were related to predictive biomarkers and additional therapeutic strategies for anti-PD-1 therapy. A number of tumor intrinsic mechanisms contribute to resistance to anti-PD-1 therapy. For example, serine/threonine kinase 11 (STK11) mutations were related to resistance to and predictions of the therapeutic efficiency of anti-PD-1 therapy in patients with lung adenocarcinomas[28,29]. The tumor microenvironment of STK11 mutations was characterized by suppressive myeloid cells and the depletion of $CD8^+$ cytotoxic T cells. The WNT/β-catenin signaling pathway is also involved in immune evasion by tumors. Specifically, the overexpression of WNT11 in tumor cells has been shown to suppress $CD8^+$ T-cell recruitment and activity by reducing the expression of CXCL10 and CCL4 through the CAMKII-mediated down-regulation of β-catenin/AFF3. Furthermore, the inhibition of CAMKII was found to enhance the efficacy of anti-PD-1 therapy in murine models of liver metastasis[30].

Galectin-1 is one of the immunosuppressive proteins targeting T-cell activities. The inhibition of galectin-1 expression by Krüppel-like factor 12 restored the infiltration and function of $CD8^+$ T cells in the

tumor microenvironment[31]. Inhibition of galectin-1 can attenuate tumor progression, facilitate T lymphocyte infiltration, and augment IFN-γ production[32]. A range of chemical and biological agents targeting galectin-1 has been developed for clinical application, demonstrating promising therapeutic efficacy[23]. However, complete remission cannot be attained through galectin-1-targeted therapy alone. Galectin-1 blockade can potentiate the immunostimulatory effects of anti-PD-1 therapy in head and neck squamous cell carcinoma[33]. In the present study, galectin-1 KO tumor cells were sensitive to the treatment by nivolumab, indicating a mechanism of pre-existing resistance induced by the expression of galectin-1, thereby corroborating previous findings that support the potential of combining galectin-1 blockade with anti-PD-1 therapy. After the administration of nivolumab, the expression of galectin-1 increased, suggesting acquired resistance to anti-PD-1 therapy. We previously reported that tetracyclines enhanced T-cell immunity by inhibiting galectin-1 secreted by tumor cells[20]. Minocycline activates anti-tumor T cell immunity by different mechanisms of PD-1 blockade. Downstream of TCR signaling, the phosphorylation of Zap70 and up-regulation of Nur77 were also found to be induced by minocycline during the early phase following T-cell activation. These changes were not observed in T cells treated with anti-PD-1 antibodies under identical conditions. These findings provide novel insights for improving cancer immunotherapy.

The present study has a number of limitations that need to be addressed. The sample size of patients who received anti-PD-1 therapy was small. Therefore, a large cohort study is needed to obtain clinical evidence for galectin-1 as the target molecule of cancer immunotherapy. A murine model investigation is also necessary to clarify the rationale for the efficacy of galectin-1 inhibition in cancer therapeutics. Previous studies and our findings have indicated the potential of combining galectin-1-targeted therapy with anti-PD-1 treatment. Further investigation into the underlying mechanism linking galectin-1 and the PD-1 pathway reinforces the clinical relevance of this combination therapy.

In conclusion, the present study indicates that galectin-1 is a predictive biomarker for the efficacy of anti-PD-1 therapy. Therapeutic strategies targeting galectin-1 may improve cancer immunotherapy.

Methods

Patient selection and data collection

This retrospective cohort study included patients with metastatic, unresectable, or postoperative recurrent NSCLC treated with anti-PD-1 monotherapy (nivolumab or pembrolizumab) at Osaka University Hospital and Osaka Toneyama Medical Center between November 2017 and August 2019. Patients with EGFR mutations or ALK translocations were excluded from the present study because they cannot be enrolled in future front-line cancer immunotherapy trials. Since this study mainly focused on galectin-1 expression, only patients with peripheral blood or biopsy specimens available before the initiation of anti-PD-1 therapy were included. The peripheral blood of enrolled patients before the initiation of anti-PD-1 therapy was subjected to a galectin-1 enzyme-linked immunosorbent assay (ELISA) and a peripheral T-cell cytotoxicity analysis. Biopsy specimens collected by bronchoscopy, computed tomographic-guided needle biopsy, or surgery from NSCLC patients before the initiation of anti-PD-1 therapy were used for immunohistochemical staining.

The following data were collected from medical charts: baseline characteristics, the treatment efficacy of anti-PD-1 therapy, and the prognosis of patients. The treatment efficacy of anti-PD-1 therapy was evaluated based on the best treatment efficacy, PFS, and overall survival (OS). The response to anti-PD-1 therapy was assessed by the attending physician based on the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1). PFS was defined as the time interval from the initiation of anti-PD-1 therapy to the date of disease progression. OS was defined as the time from the initiation of anti-PD-1 therapy to the date of death from any cause.

ELISA for the quantification of galectin-1 concentrations

The Human Galectin-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) was used to quantitatively measure the concentration of galectin-1 in the plasma of NSCLC patients or healthy donors, or in the supernatant using in vitro assays. Assays were performed according to the manufacturer's instructions.

Immunohistochemical staining

Paraffin sections of NSCLC-patient biopsy specimens were used in immunohistochemical staining for galectin-1. Immunohistochemical staining and assessments by pathologists were conducted at Morphotechnology Co., Ltd. (Hokkaido, Japan). A galectin-1 monoclonal antibody (ProteinTech Japan, Tokyo, Japan) was used as the primary antibody for staining. The dilution ratio was 1:1000 and the reaction time was one hour at room temperature. Hematoxylin-eosin staining was also performed.

Peripheral T-cell cytotoxicity assay using bispecific T cell engager (BiTE assay)

The peripheral blood mononuclear cells (PBMCs) of NSCLC patients and healthy donors were isolated from peripheral blood by gradient density centrifugation using Lymphoprep (Axis-Shield, Dundee, UK) and then subjected to T-cell assays. The construction of EphA2/CD3 BiTE was described in our previous studies[25,34]. The U251 cell line was kindly provided by Dr. Yasuko Mori (Kobe University, Japan). Cell line authentication by short tandem repeat profiling and mycoplasma testing were performed at the JCRB Cell Bank (Osaka, Japan). Wild-type (WT) or LGALS1 knockout (KO) U251 cells were plated on 96-well flat-bottomed cell culture plates (Corning, Corning, NY, USA) at a density of 1×10^4 cells per well with RPMI medium 1640 (Nacalai Tesque, Kyoto, Japan) containing 10% FBS. After a 24-hour culture at 37℃ with 5% CO_2, 5×10^4 PBMCs were added to plates with 100 ng/mL of EphA2/CD3 BiTE ± 1 μg/mL of nivolumab. After a 48-hour co-culture at 37℃ with 5% CO_2, culture supernatants were used for the galectin-1 ELISA assay. Non-adherent cells were removed by gentle washing four times with RPMI medium 1640 containing 10% FBS, and the remaining adherent viable tumor cells were detected using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2

H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA), which was performed in triplicate. The calculation of EphA2/CD3 BiTE-mediated killing was based on the degree of the reduction in viable target cells using the following formula:

% EphA2/CD3 BiTE-mediated killing = [(absorbance of non-treated wells) - (absorbance of treated wells)] / (absorbance of non-treated wells) ×100.

The generation of galectin-1 KO U251 cells is described in our previous study[20]. WT U251 cells were transfected with a galectin-1 CRISPR/Cas9 KO plasmid or control CRISPR/Cas9 plasmid (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to generate galectin-1 (LGALS1 gene) KO U251 cells or mock-transfected control U251 cells. The process of transfection with the plasmid was performed according to the manufacturer's instructions using 1 µg of plasmid DNA and 5 µL of UltraCruz® Transfection Reagent (Santa Cruz Biotechnology).

Chemical reagents

Nivolumab was provided by Ono Pharmaceutical (Osaka, Japan). A human IgG4 isotype antibody (BioLegend, San Diego, CA, USA) was used under control conditions.

Ethics approval

The present study was conducted according to the principles of the Declaration of Helsinki. The study protocol was approved by the Osaka University Hospital Ethics Committee (IRB number 13266), and written informed consent was obtained from participants before their inclusion in the study.

Statistical analysis

A two-tailed Student's *t*-test was used to examine the significance of differences between samples, with a p-value < 0.05 indicating a significant difference. PFS and OS curves were generated by the Kaplan–Meier method and compared using the Log-rank test. GraphPad Prism was used for graphing and statistical analyses (GraphPad Software, San Diego, CA, USA).

Data availability

The data generated in the present study are available within the article and its supplementary data files and upon request from the corresponding author.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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