

Siglec-7 is a predictive biomarker for the efficacy of cancer vaccination against metastatic colorectal cancer

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Abstract. Cancer immunotherapy, including vaccination, is considered a major scientific and medical breakthrough. However, cancer immunotherapy does not result in durable objective responses against colorectal cancer (CRC). To improve the efficacy of immunotherapy, the present study investigated several biomarkers for selecting patients who were expected to respond well to immunotherapy. Firstly, a comprehensive proteomic analysis was performed using tumor tissue lysates from patients enrolled in a phase II study, in which five human leukocyte antigen (HLA)-A*24:02-restricted peptides were administered. Sialic acid-binding immunoglobulin type lectin (Siglec)-7 was identified as a potential predictive biomarker. Subsequently, this biomarker was validated using western blot analysis, and immunofluorescence using tissue samples from the patients enrolled in the phase II study. The expression levels of Siglec-7 detected by immunofluorescence were quantified and their association with overall survival (OS) in patients treated with the peptide vaccine was

examined. Furthermore, considering the important role of tumor-infiltrating lymphocytes (TILs) for CRC prognosis, the densities of CD3⁺, CD4⁺, CD8⁺ and forkhead box P3 (FOXP3)⁺ T cells in CRC tissues were examined and compared with Siglec-7 expression. The mean expression levels of Siglec-7 were significantly higher in patients with poor prognosis, with an OS of ≤ 2 years, as shown in comprehensive proteomic analysis ($P=0.016$) and western blot analysis ($P=0.025$). Immunofluorescence analysis demonstrated that Siglec-7 was expressed in intratumoral macrophages. The OS in patients with high Siglec-7 expression was significantly shorter than in that in patients with low Siglec-7 expression ($P=0.017$) in the HLA-A*24:02-matched patients. However, this difference was not observed in the HLA-unmatched patients. There was no significant difference in OS between patients according to the numbers of TILs, nor significant correlation between TILs and Siglec-7 expression. In conclusion, Siglec-7 expression in macrophages in tumor tissue may be a novel predictive biomarker for the efficacy of immunotherapy against metastatic CRC.

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Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related mortality among both men and women (1). In the past decade, chemotherapy and molecular targeted treatment have improved the overall survival (OS) in patients with metastatic CRC to ~ 30 months (2). These drugs, however, have some limitations, including drug resistance and side effects, so the development of new therapeutic options to prevent

metastatic spread and eventually improve patient survival is necessary (3).

Cancer immunotherapy is considered as a major scientific and medical breakthrough (4), and several immune checkpoint-directed antibodies have increased the OS in patients with various cancers and are approved by the Food and Drug Administration (5,6). For example, PD-1 inhibitor nivolumab and pembrolizumab are used for deficient mismatch repair (dMMR) or microsatellite-instability-high (MSI-H) CRC world-wide (7,8).

However, immunotherapies, including immune checkpoint inhibitors to proficient-MMR CRC, have not achieved durable objective responses against CRC (9,10). Improving the efficacy of immunotherapies requires two approaches. One is the use of combination therapy to alter 'cold tumors' which are characterized by the absence of T cell infiltration, to 'hot tumors' characterized by the accumulation of proinflammatory cytokines and T cell infiltration (5,11). The other is the identification of biomarkers to select patients who are expected to respond well to immunotherapy.

The authors of the present study previously reported phase I and II studies in which five epitope peptides were administered to advanced-stage CRC patients (12,13). In these studies, a low neutrophil/lymphocyte ratio and a low plasma interleukin (IL)-6 level were the potential markers for improved survival time of vaccinated patients (14,15). Furthermore, it was also shown that several miRNAs and the integrity of plasma cell-free DNA were predictive biomarkers for active immunotherapy using epitope peptides (15-18).

This study aimed to identify novel predictive biomarkers to select patients who are highly responsive to immunotherapy to improve the efficacy of immunotherapy. To this end, a comprehensive analysis of proteins in tumor tissues was performed and sialic acid-binding immunoglobulin type lectin (Siglec)-7 was identified as a potential predictive biomarker for immunotherapy.

Siglecs are a family of transmembrane receptors predominantly found in both innate and adaptive immune cells, involved in distinguishing between self and non-self-cells by recognizing sialic acids at the cellular surface (19,20). Siglec-7, the seventh member of the Siglec family, is mainly expressed on natural killer (NK) cells, monocytes, macrophages, and a minor subset of CD8⁺ T cells (21,22), and acts as an inhibitory receptor. The cytoplasmic portion of Siglec-7 contains immune receptor tyrosine-based inhibition motifs (ITIMs), which provide inhibitory signals by recruiting the SH2-domain-containing tyrosine phosphatase (SHP)-1 and SHP2 (22). SHP1 and SHP2 inhibit NK cell activation pathways such as the NKG2D pathway, suppressing NK cell cytotoxicity to tumor cells (23). However, it has never been evaluated for its possible role in cancer immunotherapy. In the present study, Siglec-7 was evaluated for its potential role as a novel biomarker for active immunotherapy.

Materials and methods

Summary of the phase II study. To assess the clinical benefits of cancer vaccination treatment, a phase II study was conducted using five human leukocyte antigen (HLA)-A*24:02-restricted peptides, including kinase of the outer

chloroplast membrane 1 (KOC1) (24), translocase of outer mitochondrial membrane 34 (TOMM34) (25), ring finger protein 43 (RNF43) (26), vascular endothelial growth factor receptor (VEGFR) 1 and 2 (27,28). This phase II study was a non-randomized, HLA-A status double-blind study. The detailed protocol of this phase II study was previously described (13). Briefly, the therapy consisted of a cocktail of five therapeutic epitope peptides in addition to oxaliplatin-containing chemotherapy. The cocktail containing 3 mg of each of the five peptides was mixed with 1.5 ml of incomplete Freund's adjuvant and administered subcutaneously into the thigh or axilla regions every week for 13 weeks, followed by the vaccination once every 2 weeks. Patients ≥ 20 years old with histologically confirmed advanced CRC who were chemotherapy-naïve, who had adequate functions of critical organs, and had a life expectancy of ≥ 3 months were eligible. Between February 2009 and November 2012, 96 chemotherapy-naïve CRC patients were enrolled with masked HLA-A*24:02 status.

Sample collection. From the 96 patients who were enrolled in the phase II trial (50 were HLA-A*24:02-matched and 46 were unmatched), 63 formalin-fixed paraffin-embedded (FFPE) tissue samples of primary CRC were obtained (32 were HLA-A*24:02-matched and 31 were unmatched) (Fig. 1). In 14 of the 32 HLA-A*24:02-matched patients, fresh tissues were also snap-frozen in liquid nitrogen and preserved at -80°C until further examination. Primary CRC tissues were obtained by surgery prior to the vaccine treatment at Yamaguchi University Hospital and affiliated hospitals. All samples were obtained with the patients' written informed consent. This study was conducted according to the Declaration of Helsinki and was approved by the Institutional Ethics Review Boards of Yamaguchi University (approval no. H20-102; Clinical Trials Registry: UMIN000001791).

Comprehensive proteomic analysis of tumor tissue. A comprehensive analysis of the protein levels in tumor tissue lysate was performed using the SOMAscan (SomaLogic, Inc.) to quantify 1,129 biologically relevant proteins as previously described (29). Frozen CRC tissue samples were available from patients who survived for either more than three years or less than two years (Fig. 1). According to the manufacturer's protocol (SomaLogic, Inc.), the total protein of the frozen CRC tissue sample was extracted with lysis buffer T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) through a Qiagen TissueLyser (Qiagen). Samples were sent to SomaLogic and analyzed using the SOMAscan assay. In this assay, protein signals were converted to nucleotide signals using chemically modified nucleotides so that quantification could be done using relative fluorescence signal on microarrays. For this reason, SOMAscan measurements were presented as relative fluorescence units (RFUs).

Western blot analysis. Western blot analysis was performed as previously described (30), using the same extracts as those used in the comprehensive analysis of SOMAscan. Briefly, protein samples (10 μg) were separated on 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc.).

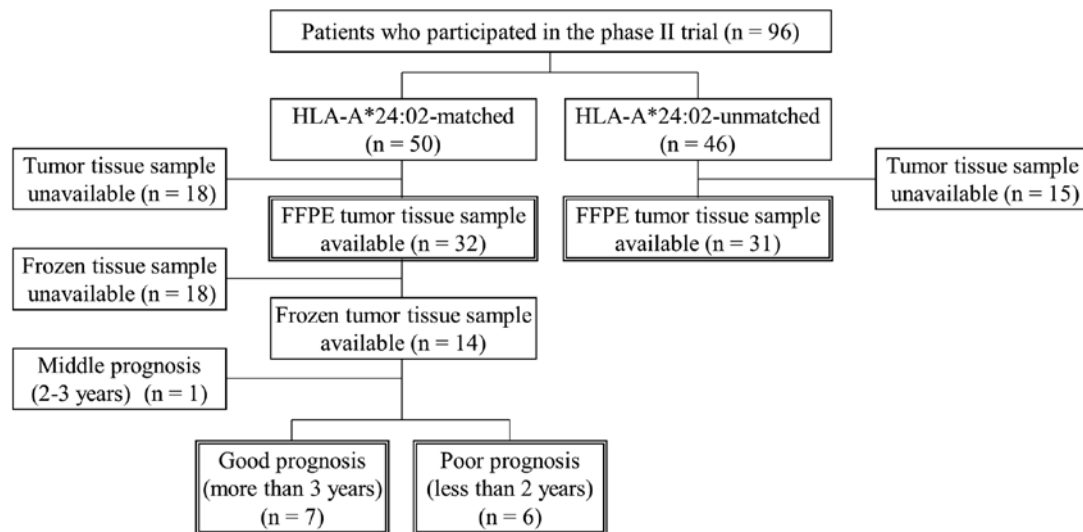


Figure 1. CONSORT diagram of the analyses in this study. Thirteen frozen tissue samples from HLA-A*24:02-matched patients were analyzed using SOMAscan, the comprehensive proteomic analysis; and 63 FFPE tumor tissue samples, 32 HLA-matched and 31 HLA-unmatched patients, were analyzed using immunofluorescence. HLA, human leukocyte antigen; FFPE, formalin-fixed paraffin-embedded.

Membranes were blocked by pre-incubation with 3% skim milk for 30 min at room temperature and then were incubated with anti-Siglec-7 antibody (ProteinTech Group, Inc.) at 4°C overnight. After washing 3 times with Tris-buffered saline with Tween-20 (TBST) buffer, the membranes were incubated with the corresponding secondary antibody for 1 h at room temperature. Immunoreactions were detected using an enhanced chemiluminescence (ECL) western blotting detection system and an Amersham Imager 600 (GE Healthcare Life Sciences). Densitometry analysis was performed using ImageJ software (National Institutes of Health) (31). Since the protein levels of VCP, one of the housekeeping proteins, are more stable compared to other housekeeping proteins, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin, VCP was chosen as the loading control (30,32,33).

Immunohistochemistry. Immunohistochemistry was carried out on 4- μ m-thick FFPE sections. For staining Siglec-7, sections were deparaffinized through xylene and graded alcohols, and antigen retrieval was performed in 10 mM Tris-EDTA buffer pH 9.0 (Dako) in a microwave at 95°C for 20 min. Endogenous peroxidase activity in the sections was blocked with 3% hydrogen peroxidase for 20 min, and nonspecific protein binding was blocked with Protein Block Serum-Free (Dako) for 10 min. The staining procedures were performed in a Dako Autostainer (Dako) according to the manufacturer's protocol. Sections were incubated with an anti-Siglec-7 antibody (rabbit polyclonal, 13939-1-AP, ProteinTech Group, Inc.; dilution 1:800) at room temperature for 1 h. After washing 3 times with phosphate-buffered saline (PBS), the sections were incubated with the corresponding secondary antibody for 30 min. The reactions were visualized with 3,3'-diaminobenzidine chromogen (DAB; Dako) and counterstained with Mayer's hematoxylin. Images were acquired using the All-in-one fluorescence microscope BZ-X710 (Keyence).

Considering the important role of tumor-infiltrating lymphocytes (TILs) for the CRC prognosis, the densities of CD3⁺, CD4⁺, CD8⁺, and forkhead box P3 (FOXP3)⁺ T cells

in CRC tissues were also examined. Immunohistochemistry for TILs was performed as previously described (34,35). Briefly, using the Ventana Discovery XT staining system (Ventana), the sections were incubated with anti-CD3 antibody (mouse monoclonal, 518110079; Ventana), anti-CD4 (mouse monoclonal, 518108816; Ventana), anti-CD8 (mouse monoclonal, IR623; Dako; dilution 1:50), and anti-FOXP3 (mouse monoclonal, ab20034; Abcam; dilution 1:100). The microscopic images were acquired using a high-resolution digital slide scanner NanoZoomer-XR C12000 (Hamamatsu Photonics).

Immunofluorescence. Immunofluorescence was carried out on 4- μ m-thick FFPE sections the same way as immunohistochemistry. Sections were deparaffinized and antigen retrieval was performed in 10 mM Tris-EDTA buffer pH 9.0 (Dako) in a microwave at 95°C for 20 min. Nonspecific protein binding was blocked with Protein Block Serum-Free (Dako) for 10 min. Sections were incubated with an antibody mixture (1:800 diluted anti-Siglec-7 antibody, and 1:400 diluted anti-CD68 antibody; mouse monoclonal, Ab783; Abcam) at 4°C overnight. The next day, after washing 3 times with PBS, sections were incubated with secondary antibody mixture (1:1,000 diluted anti-mouse Alexa Fluor 568 and 1:1,000 diluted anti-rabbit Alexa Fluor 488; Thermo Fisher Scientific) for 60 min at room temperature. Slides were counterstained with DAPI blue to visualize nuclei. All staining procedures were performed manually, and stained sections were visualized and photographed using the All-in-one fluorescence microscope BZ-X710 (KEYENCE; magnification, x200). From each section, 10 fields near the center of the tumor with the highest density of Siglec-7-positive cells and CD68-positive cells were manually selected by observers. Images were analyzed with an algorithm for positive pixel count using ImageJ software (NIH) to quantify the expression levels of Siglec-7 and CD68. The threshold intensity was set at 40 for Siglec-7 and CD68 staining. The results were presented as a percent of the total positive area to the area of the examined fields.

Table I. Predictive markers from comprehensive proteomic analysis of tumor tissue.

Rank	Target protein	Good prognosis (n=7)		Poor prognosis (n=6)		lLog ₂ ratio	Fisher ratio	Welch's t-test
		Mean	SD	Mean	SD			P-value
1	Sonic Hedgehog	492.5	226.9	230.3	82.7	1.1	3.66	0.022
2	ICOSLG	14492.5	14465.9	41296.1	34872.0	1.5	2.29	0.089
3	Lysozyme	9204.3	5769.5	19217.0	12240.3	1.1	2.20	0.079
4	Siglec-7	934.8	511.9	2272.1	1121.8	1.3	2.00	0.016
5	Siglec-9	416.4	167.9	1158.8	1044.2	1.5	1.62	0.089
6	Fibronectin	4794.1	3336.5	13751.4	8483.4	1.5	1.60	0.025
7	FCGR3B	1494.4	827.5	3934.3	3370.6	1.4	1.42	0.089
8	TIMP1	6566.0	4235.2	14537.2	11608.0	1.1	1.32	0.117
9	LBP	3525.2	2064.2	12051.2	14610.8	1.8	1.22	0.152
10	C1q	14432.5	10855.0	32501.8	22469.4	1.2	1.20	0.085

Proteins eligible for predictive biomarkers were narrowed down by the absolute log₂ ratio ≥ 1 and ranked according to the Fisher ratio between the good and poor prognosis groups. Good prognosis, with overall survival of 3 years or more; poor prognosis, with overall survival of 2 years or less; SD, standard deviation; ICOSLG, inducible T cell costimulator ligand; Siglec-7, sialic acid-binding immunoglobulin-like lectin 7; Siglec-9, sialic acid-binding immunoglobulin-like lectin 9; FCGR3B, Fc fragment of IgG receptor IIIb; TIMP1, tissue inhibitor of metalloproteinase 1; LBP, lipopolysaccharide binding protein; C1q, complement component 1, q subcomponent.

Measurement of TILs. Based on the immunohistochemistry for TILs, the number of TILs was measured as previously described (34,35). Briefly, intratumoral-infiltrating CD3⁺, CD4⁺, CD8⁺ and FOXP3⁺ cells were defined as TILs and their numbers were measured. Those found in the peritumoral stroma and extratumoral lymphoid structures were excluded from this analysis. A computerized image analysis system Tissue Studio (Definiens) was used to score all tumor lesions. The numbers of TILs were recorded in square millimeters as the mean number of positive cells per tumor tissue unit.

Statistical analysis. In comprehensive protein analysis, differential expression of proteins was detected using the log₂ and Fisher ratio using Microsoft Excel 2010 (Microsoft Corporation) (36). The log₂ ratio for a protein k was calculated according to the following formula:

$$\log_2 \text{ ratio} = \log_2 \left(\frac{\bar{x}_k(\text{good prognosis group})}{\bar{x}_k(\text{poor prognosis group})} \right),$$

where \bar{x}_k is the k^{th} protein of the sample mean of the good or poor prognosis group. The Fisher ratio F for a protein k was calculated using the following formula:

$$\text{formula: } F(k) = \frac{[\bar{x}_k(\text{good prognosis group}) - \bar{x}_k(\text{poor prognosis group})]^2}{\frac{1}{2}[s_k^2(\text{good prognosis group}) + s_k^2(\text{poor prognosis group})]},$$

where s_k^2 is the k^{th} protein of the sample variance of the good or poor prognosis group.

Differences between the two groups were estimated using the Welch's t-test, which was selected for this study because recent statistical recommendations and simulation studies suggest using this test under either homoscedasticity or heteroscedasticity conditions (37). The categorical variables were compared using the χ^2 or Fisher's exact tests. The strength

of a correlation between two groups was assessed by the Spearman's rank correlation coefficient. The optimal cut-off values of the expression levels of Siglec-7, CD3, CD4, CD8, and FOXP3 were determined using either the median value or the time-dependent receiver operating characteristic (ROC) curve analysis using the Kaplan-Meier (KM) estimation method and Youden's index (sensitivity + specificity - 1) (38). The survival curves were estimated using the KM method and tested using the log-rank test. All statistical analyses were performed using R language for 64-bit Windows (version 3.6.1, R Development Core Team). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Selection of candidate protein to predict the efficacy of vaccination. Comprehensive analysis of the expression profiles of 1,129 proteins in 13 frozen CRC tissue samples from HLA-A*24:02-matched patients was performed. The patients were divided into good and poor prognosis groups; in 7 cases with good prognosis, the patients had OS of 3 years or more and in 6 cases with poor prognosis, the patients had OS of 2 years or less. Comparing the protein expression levels of the two groups, 23 proteins satisfied the absolute log₂ ratio ≥ 1 and the Fisher ratio ≥ 1 . Of the 23 proteins, Table I shows the 10 proteins with the highest Fisher ratio. The expression level of Sonic hedgehog (SHH) in the good prognosis group was significantly higher than that in the poor prognosis group ($P = 0.022$). In contrast, the expression levels of Siglec-7 and fibronectin were significantly higher in the poor prognosis group than those in the good prognosis group ($P = 0.016$ and 0.025 , respectively). Among them, Siglec-7 was selected as a candidate protein because of the lowest P-value.

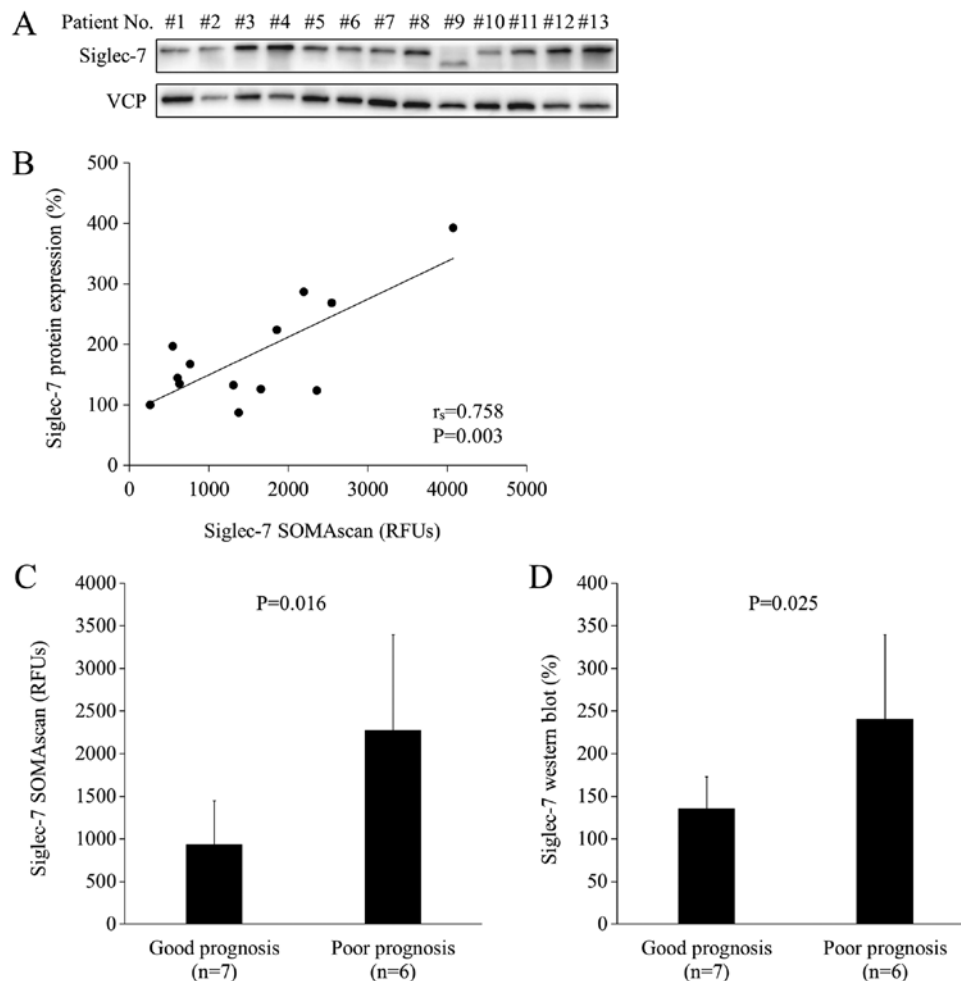


Figure 2. Confirmation of Siglec-7 expression levels and their correlation with patient prognosis. (A) The same protein samples as those in the comprehensive analysis were used in western blot analysis (n=13). The lower protein band in lane #9 may reflect higher expression of the shorter isoform of Siglec-7 in the tumor tissue of patient #9 compared with the other patients. In subsequent analysis, protein bands were quantified by densitometry and the results were presented as relative expression levels compared to the Siglec-7 expression level in patient number 1 (=100%). (B) The levels of Siglec-7, as measured by SOMAscan and western blot analysis, were correlated (n=13). (C) The levels of Siglec-7 measured using the SOMAscan analysis were significantly higher in the poor prognosis group. (D) The levels of Siglec-7 measured using western blot analysis were significantly higher in the poor prognosis group. Siglec-7, sialic acid-binding immunoglobulin-like lectin 7; VCP, valosin containing protein; RFU, relative fluorescence unit; OS, overall survival.

Confirmation of candidate protein expression using western blot analysis. To validate the results obtained in comprehensive analysis of SOMAscan, western blot analysis was performed using the same 13 samples as the ones used in SOMAscan analysis (Fig. 2A). As shown in Fig. 2A, the protein band in lane #9 was lower than those in the other lanes. Siglec-7 has three isoforms, and the shorter isoform may have been highly expressed in the tumor tissue of patient #9 compared with the other patients. The levels of Siglec-7 showed a positive correlation between SOMAscan measurements and western blot measurements ($r_s=0.758$, $P=0.00268$; Fig. 2B). The mean expression levels of Siglec-7 were significantly higher in the poor prognosis group based on both SOMAscan analysis ($P=0.016$) and in western blot analysis ($P=0.025$) (Fig. 2C and D). These results indicated that the levels of Siglec-7 protein in the CRC tissue were significantly higher in patients with poor prognosis than in those with good prognosis in HLA-A*24:02-matched cohort.

Localization of Siglec-7 in tumor tissue. To identify the localization of Siglec-7 in CRC tissue, immunohistochemistry and

immunofluorescence were performed. Immunohistochemistry showed that Siglec-7 was expressed in stromal cells located between or around tumor cells (Fig. 3A). Immunofluorescence showed that Siglec-7 was expressed in stromal cells which also expressed CD68 (Fig. 3B). These results indicated that Siglec-7 was expressed in intratumoral macrophages.

Validation of Siglec-7 as a predictive biomarker of vaccination. The levels of Siglec-7 expression in 63 CRC tissue samples from 32 HLA-A*24:02-matched patients and 31 HLA-A*24:02-unmatched patients were examined using immunofluorescence (Fig. 1; Table II). The levels of Siglec-7 expression ranged from 0.00001 to 7.81% (median, 0.0279%), and from 0.0400 to 0.457% (median, 0.120%) in HLA-A*24:02-matched and -unmatched patients, respectively. The comprehensive proteomic analysis in the present study was based on the survival of stage IV patients. Since the median OS among stage IV CRC patients is approximately 3 years, the optimal cut-off value was determined using ROC curve analysis at 36 months. This analysis was performed in HLA-A*24:02-matched patients because HLA-restricted

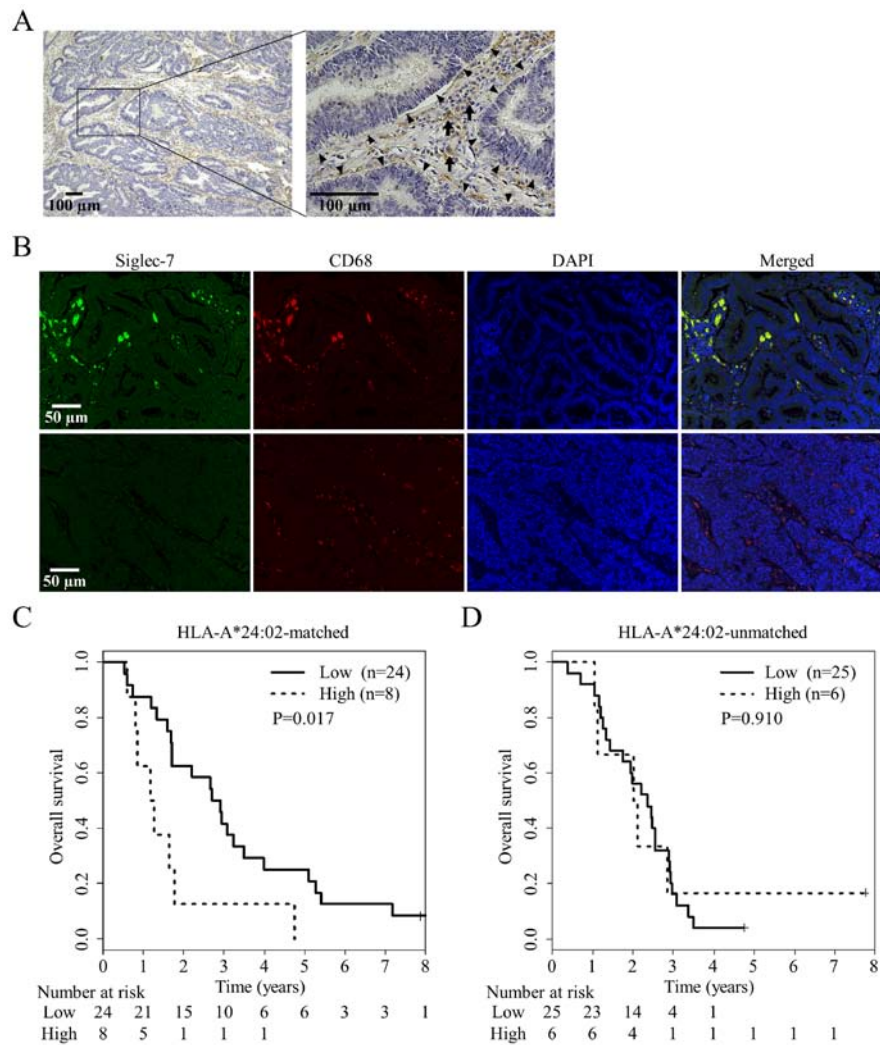


Figure 3. Siglec-7 expression in tumor microenvironment and its correlation with patient prognosis. (A) Stromal cells in the tumor area expressed Siglec-7 (indicated by arrows), whereas tumor cells did not (indicated by arrowheads). They were defined based on their locations and morphological findings. Scale bar, 100 μ m. (B) Representative immunofluorescence images of Siglec-7 (green), CD68 (red), and nuclei (blue) in CRC tissue. Almost all of the Siglec-7⁺ cells expressed CD68 simultaneously, whereas there were some cells expressing only CD68. The upper row represents a tumor with high expression level of Siglec-7, and the lower one shows a tumor with low expression. Scale bar, 5 μ m. (C) Overall survival in patients with high levels of Siglec-7 expression as detected with immunofluorescence images was significantly ($P=0.017$) shorter than that in patients with low levels of Siglec-7 expression in HLA-A*24:02-matched group. (D) There was no significant difference in overall survival between patients with high and low levels of Siglec-7 expression in HLA-A*24:02-unmatched patients. Siglec-7, sialic acid-binding immunoglobulin-like lectin 7; HLA, human leukocyte antigen; CRC, colorectal cancer; RFUs, relative fluorescence units.

peptides vaccines are theoretically effective for these patients. The cut-off value was presented as a percentage of the total positive area of Siglec-7 to the area of the examined fields. A percent of 0.213 was selected as the cut-off value for Siglec-7 expression. In the HLA-A*24:02-matched patients, the OS in patients with high Siglec-7 expression was significantly shorter than that in patients with low Siglec-7 expression ($P=0.017$; Fig. 3C). In contrast, in the HLA-A*24:02-unmatched patients, there was no significant difference in OS between patients with high or low Siglec-7 expression ($P=0.910$; Fig. 3D). In patients with low Siglec-7 expression, there was a significant difference in OS between HLA-A*24:02-matched and -unmatched patients ($P=0.041$; Fig. S1A), whereas there was no significant difference in patients with high Siglec-7 expression ($P=0.179$; Fig. S1B). The levels of Siglec-7 expression in tumor tissue were correlated with that of CD68 ($rs=0.786$, $P<0.001$; Fig. S2A). However, there was no significant difference in OS between patients with high and low levels of CD68 expression in

HLA-A*24:02-matched patients ($P=0.528$; Fig. S2B). These results indicated that Siglec-7 expression in tumor microenvironment might be a predictive biomarker of the efficacy of cancer vaccine therapy.

Relationship of TIL infiltration and prognosis with Siglec-7 expression. Because TILs have been reported as biomarkers for CRC, they were analyzed using immunohistochemistry in CRC tissue samples from 32 HLA-A*24:02-matched patients, the same as those used for Siglec-7 analysis (Fig. S3). Using ROC curve analysis at 36 months, the optimal cut-off values were determined as 440.1, 133.8, 52.6 and 17.8 for CD3⁺, CD4⁺, CD8⁺ and FOXP3⁺ cell densities, respectively. There was no significant difference in OS between patients with high and low numbers of TILs including CD4⁺, CD8⁺ and FOXP3⁺ T cells ($P=0.319$, 0.605 and 0.242, respectively; Fig. 4), although there was a trend for better OS in patients with high infiltration of CD3⁺ lymphocytes ($P=0.065$; Fig. 4A). Next, the correlation

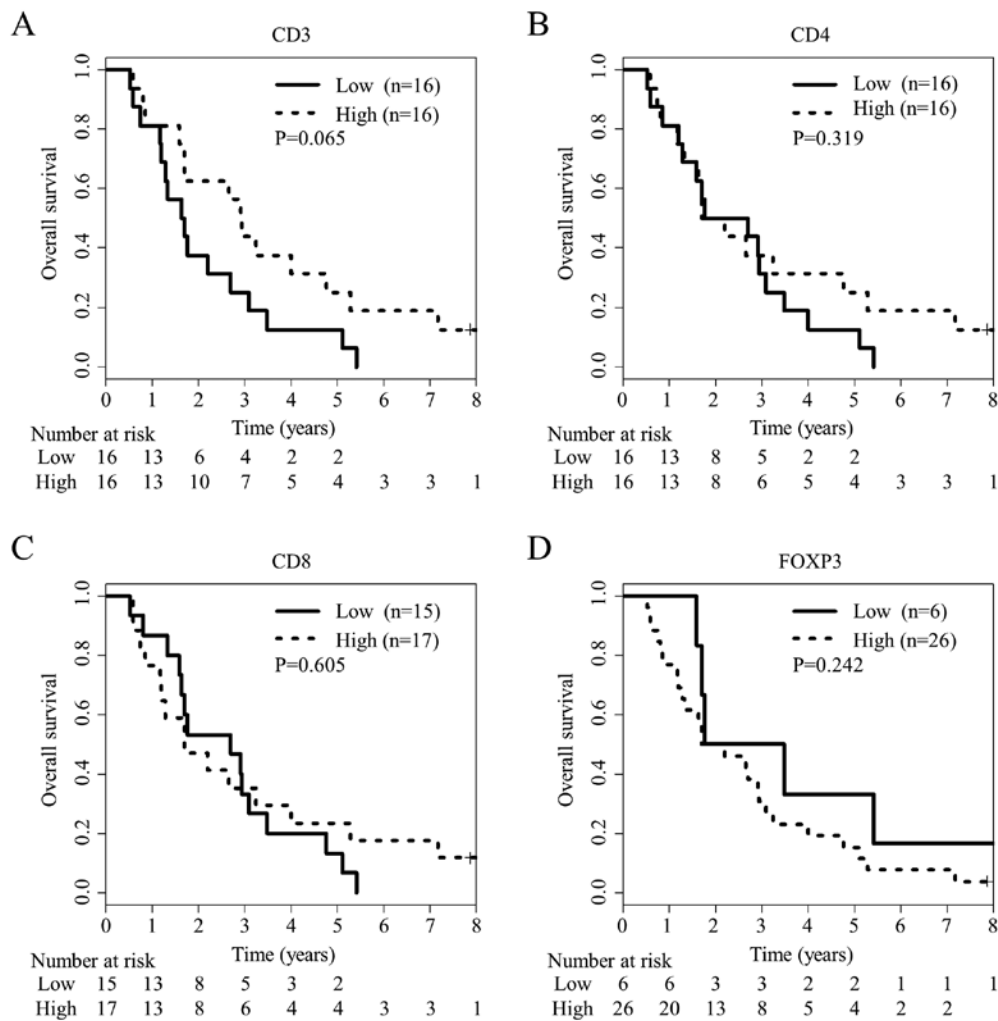


Figure 4. Overall survival according to the number of tumor-infiltrating lymphocytes (TILs). Kaplan-Meier curves for overall survival according to the number of CD3, CD4, CD8 and FOXP3 are shown. There was no significant difference in overall survival between patients with high or low numbers of TILs. (A) CD3, (B) CD4, (C) CD8, and (D) FOXP3. FOXP3, forkhead box P3.

between Siglec-7 expression and TILs in CRC tissues was examined. There were no significant associations between the levels of Siglec-7 expression detected in immunofluorescence and the numbers of CD3⁺, CD4⁺, CD8⁺ and FOXP3⁺ T cells in immunohistochemistry ($P=0.565$, 0.154 , 0.982 and 0.676 , respectively; Fig. 5). These findings indicated that lymphocytes and monocytes/macrophage infiltration might be independent.

Discussion

The purpose of the present study was to explore proteins as novel biomarkers to predict the efficacy of immunotherapy before treatment. First, it was demonstrated that high levels of Siglec-7 expression in tumor tissues were associated with shorter OS in patients treated with peptide vaccines for metastatic CRC. Second, it was shown that Siglec-7 was expressed in macrophages in CRC tissue. Further, there was no significant correlation between the level of Siglec-7 expression and the number of TILs in CRC tissue. These results indicated that high levels of Siglec-7 expression in intratumoral macrophages can be a negative biomarker of the vaccine treatment efficacy against metastatic CRC. To our knowledge, this is the first report showing the relationship between Siglec-7 and CRC prognosis.

In the comprehensive proteomic analysis, the good and poor prognosis groups showed significant differences in expression levels of Siglec-7, SHH, and fibronectin. SHH is a ligand for the Hedgehog signaling pathway, which is critical for embryonic development and carcinogenesis (39). Although increased expression of SHH has been associated with poor prognosis in patients with various malignancies, including CRC (40,41), the present study obtained opposite results in this aspect. Furthermore, fibronectin is a ligand for many members of the integrin receptor family and it is involved in cell adhesion, migration, growth, and differentiation (42). Because the relationship between fibronectin and CRC has been already reported (43,44), it was difficult to find additional roles for this protein as a biomarker in cancer vaccination against CRC. For these reasons, SHH and fibronectin were excluded as candidates for predictive biomarkers.

Low levels of Siglec-7 expression in tumor tissue was associated with better prognosis in HLA-A*24:02-matched patients, but not in the unmatched patients. HLA-restricted epitope peptides show theoretical antitumoral effects only in HLA-matched patients. And only HLA-A*24:02-matched patients were considered to be treated with vaccines in the present study. Therefore, the resulting difference in OS based

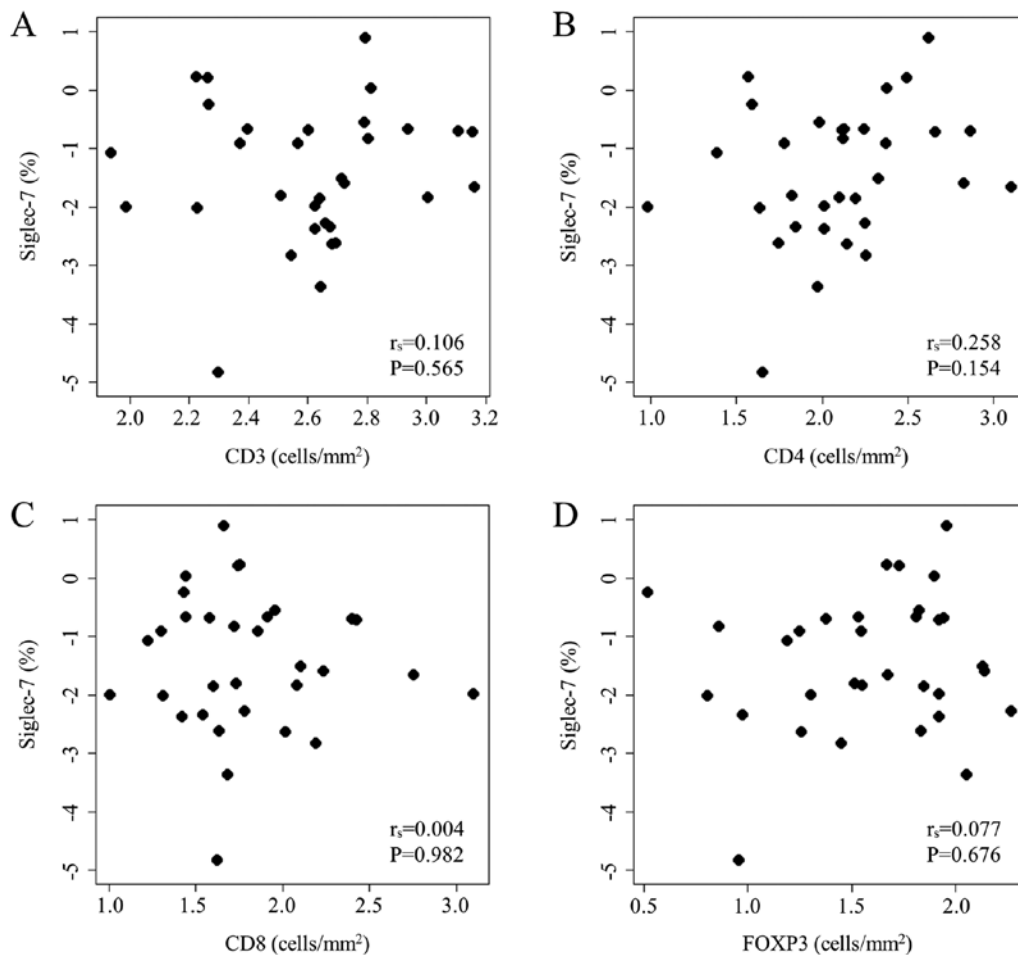


Figure 5. Relationship between Siglec-7 expression and the number of tumor-infiltrating lymphocytes (TILs). Scatterplots for CD3, CD4, CD8 and FOXP3 versus Siglec-7 are shown. The values were transformed by \log_{10} . There was no significant correlation between the levels of Siglec-7 expression and the numbers of TILs. (A) CD3, (B) CD4, (C) CD8, and (D) FOXP3. Siglec-7, sialic acid-binding immunoglobulin-like lectin 7; FOXP3, forkhead box P3.

on Siglec-7 expression was only in HLA-A*24:02-matched patients, indicating that Siglec-7 was not a prognostic marker for CRC but a predictive biomarker for cancer vaccination.

Siglec-7, a member of the CD33-related Siglecs, is mainly expressed in NK cells and monocytes/macrophages (22). The distribution of Siglec-7⁺ cells has been reported to differ between peripheral blood and colonic lamina propria (45). In the peripheral blood, 75% of Siglec-7⁺ cells were NK cells and 8% were monocytes. In colonic lamina propria, in contrast, 76% of Siglec-7⁺ cells were monocyte/macrophage lineages and 4% were NK cells. In this study, Siglec-7 was observed mostly in CD68⁺ cells in CRC tissue, thereby it was suggested that intratumoral macrophages expressed Siglec-7. The role of Siglec-7 in macrophage has been poorly explored, whereas Siglec-9, another CD33-related Siglec that shares 84% sequence homology with Siglec-7, was reported to play an inhibitory role in macrophages (46). Specifically, Siglec-9 mediated reduction in proinflammatory cytokine tumor necrosis factor (TNF)- α production and potent increment in anti-inflammatory cytokine IL-10 production via ITIMs (47). Therefore, it was hypothesized that Siglec-7-expressing macrophages may mediate the reduction in secretion of proinflammatory cytokine TNF- α and increase in secretion of anti-inflammatory cytokine IL-10, resulting in immunosuppression of the tumor microenvironment. MSI status, another factor related to the

tumor microenvironment, was also analyzed in the present study, and only one patient had MSI-high CRC (data not shown). Although the level of Siglec-7 expression was low in the MSI-high CRC, the relationship between Siglec-7 expression in CRC tissue and MSI status was not analyzed because it was statistically inappropriate.

Cancer vaccination shows antitumoral effects by introducing tumor antigen-specific cytotoxic T lymphocytes (CTLs). Described as the cancer-immunity cycle (48), injected HLA-restricted epitope peptides are captured and presented to T cells by dendritic cells via HLA molecules. Then, activated tumor antigen-specific CTLs infiltrate the tumor, recognizing and killing target cancer cells. However, CTLs may have their function inhibited by PD-L1 and immunosuppressive mediators such as IL-10 and transforming growth factor- β in the tumor microenvironment (49,50). Siglec-7 may pose an obstacle to CTLs by mediating immunosuppression of tumor microenvironment via regulation of TNF- α and IL-10 secretions, resulting in suppressed efficacy of vaccine treatment against metastatic CRC. These mechanisms may explain the association between high levels of Siglec-7 expression in intratumoral macrophages and poor prognosis in HLA-A*24:02-matched patients.

TILs, especially CD3⁺ and CD8⁺ T cells, are prognostic biomarkers for CRC (28,51). For instance, a scoring system

Table II. Characteristics of patients in the phase II study whose tissues were analyzed by immunofluorescence.

Characteristics	HLA-A*24:02		P-value
	Matched (n=32)	Unmatched (n=31)	
Age			
Mean	67.9	64.3	0.069
Range	47-82	47-77	
Sex			
Male	13	18	0.211
Female	19	13	
Unresectable site			
Liver	18	24	0.300
Lung	11	9	
Dissemination	3	2	
Bone	0	2	
Lymph node	3	9	
Other	3	1	
Number of metastatic organs			
One	26	19	0.068
Two	6	8	
Three	0	4	
Location of tumor			
Colon	22	24	0.572
Rectum	10	7	

HLA, human leukocyte antigen.

based on CD³⁺ and CD8⁺ T cells densities within the tumor and its invasive margin, the immunoscore, was demonstrated to be a strong prognostic factor for CRC patients (52,53). In the present study, Siglec-7 expression was not associated with CD3⁺, CD4⁺, CD8⁺ and FOXP3⁺ T cells. It was suggested that Siglec-7 was an independent biomarker from TILs. The analysis of Siglec-7 might have led to these results by assessing macrophages rather than lymphocytes in the tumor microenvironment.

The present study, however, had several limitations. The first one is the small number of patients enrolled in this study. Second, multivariate analysis, including clinicopathological factors to adjust for confounding factors, was not performed because it was statistically inappropriate due to the small number of patients. The third limitation concerns the lack of mechanistic studies. Nonetheless, understanding the functions of Siglec-7 in the tumor environment might lead to novel immunotherapeutic strategies such as the alteration of cold tumor to hot tumor. For example, because Siglecs are endocytic receptors suitable for drug delivery, the alteration may be achieved by administering a Siglec-7-specific antibody conjugated to toxins or chemotherapeutic agents to deplete Siglec-7-expressing macrophages (54). Finally, the relationship between Siglec-7 expression and other immunologically

important molecules including PD-1, PD-L1 and HLA expressions were not evaluated.

In conclusion, Siglec-7 expression in macrophages in tumor tissue might be a novel predictive biomarker for the efficacy of immunotherapy against metastatic CRC. Further studies are needed to confirm the utility of Siglec-7 as a predictive biomarker and to analyze the role of Siglec-7 in the tumor microenvironment.

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Availability of data and materials

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

Authors' contributions

KY, SH and HN designed the study. KY, NS, MX, NF, RT, SY, STo, SM, HM, YT, SK, YS, YW, MI, STa, TI, TU, YHo, HK, TF and YK contributed to patient recruitment and collection of data, and analysis and interpretation of data. KY, YN, HO and YHa performed the statistical analysis. KY, SH and HN wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was carried out according to the Declaration of Helsinki on experimentation on human subjects and was approved by the Institutional Ethics Review Boards of Yamaguchi University (approval number: H20-102; Clinical Trials Registry: UMIN000001791). Written informed consent for participation in this study was obtained from each patient.

Patient consent for publication

Written informed consent for publication was obtained from each patient at the time of enrollment.

Competing interests

The authors declare that they have no competing interests.

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