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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH

Clinical Significance of SIRPα Expression on Tumor-Associated Macrophages in Patients with Lung Squamous Cell Carcinoma

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ABSTRACT

Background. Signal-regulatory protein alpha (SIRPα) is an immune checkpoint molecule expressed on macrophages that functions to inhibit phagocytosis by binding to CD47 expressed on tumor cells. SIRPα has attracted increasing attention as a novel target for cancer immunotherapy; however, the expression and immune function of SIRPα in lung squamous cell carcinoma (LUSC) remain unclear. Therefore, this study aimed to identify the clinical importance of SIRPα expression in LUSC and to explore the factors that elevate SIRPα expression.

Patients and Methods. Primary LUSC specimens surgically resected from 172 patients underwent immunohistochemical evaluation of the association of SIRPα expression on tumor-associated macrophages with clinicopathological features and clinical outcomes. Furthermore, we analyzed the association of SIRPα expression with tumor-infiltrating lymphocytes and the expression of programmed cell death ligand 1 (PD-L1). In vitro, monocytes were treated with cytokines, and SIRPα protein expression was assessed by flow cytometry.

Results. There were no differences in SIRPα expression and clinicopathological factors. High SIRPα expression was significantly associated with PD-L1-positive expression, and high CD8, PD-1, and CD163 expression. The high SIRPα expression group showed significantly shorter recurrence-free survival (RFS) and overall survival (OS). On multivariate analysis, high SIRPα expression was an independent poor prognostic factor for RFS and OS. The expression of SIRPα protein in monocytes was upregulated by treatment with IFNγ.

Conclusion. Our analysis revealed that high SIRPα expression significantly predicts poor prognosis in patients with surgically resected LUSC.

Keywords SIRPα · Lung cancer · PD-L1 · Prognostic factor · Squamous cell carcinoma

Lung cancer is a significant global health issue and the primary cause of cancer mortality worldwide.[1,2] Surgery is the main treatment of choice for early-stage non-small cell lung cancer (NSCLC), while multimodality treatment, including radiation therapy and chemotherapy, is used for lung cancer in advanced stages. Recently, immunotherapy and molecular drugs targeting driver gene mutations have shown excellent results, promoting a significant shift in the treatment approach.[3–6] Lung squamous cell carcinoma (LUSC) is the second most prevalent histological type of NSCLC, after adenocarcinoma, making up 20–30% of all lung cancer cases.[7,8] LUSC is usually found in older patients with advanced disease progression and metastases at diagnosis. In addition, LUSC has a limited availability of molecular targeted drugs.[9–11] Therefore, patients with LUSC tend to

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have a poorer prognosis compared with other histological types of NSCLC.[7]

Immunotherapy has been demonstrated to be highly successful in the treatment of cancer.[12,13] Tumor cells avoid immune detection and promote tolerance by establishing immune checkpoints. In particular, the programmed cell death ligand 1 (PD-L1) on tumor cells enables immune escape by binding to programmed cell death-1 (PD-1) on activated cytotoxic T lymphocytes (CTLs).[14] Subsequently, immune checkpoint inhibitors activate a previously stalled antitumor immune response. Nevertheless, immunotherapy with anti-PD-1/PD-L1 antibodies is effective in only 30% of patients with NSCLC patients.[3,15] Therefore, although immunotherapy has made great strides, response rates remain low. It is essential to create innovative personalized treatment strategies for patients with LUSC.[8,16]

Recently, there has been an increasing interest in targeting signal-regulated protein alpha (SIRPα) and cluster of differentiation (CD) 47 as a potential therapy for cancer.[17,18] SIRPα, a type I transmembrane glycoprotein found on dendritic cells, monocytes, and macrophages, is an immune checkpoint molecule that binds to CD47, which is expressed on neurons, erythrocytes, and tumor cells, resulting in the inhibition of phagocytosis. Inhibition of macrophage phagocytosis by this axis is known as the "don't eat me" signal.[19–21]

Tumor shrinkage through suppression of the SIRPα/CD47 system has been observed in mouse models of solid tumors, including breast cancer, malignant melanoma, and kidney cancer.[20,22] Furthermore, previous studies have investigated the significance of SIRPα expression in malignant tumors and found that high SIRPα expression was associated with a negative prognosis.[23,24] However, few reports have examined the role of SIRPα in LUSC. Therefore, this study aimed to determine the clinical significance of SIRPα protein expression in LUSC and to explore the factors that elevate SIRPα expression.

METHODS

Patients and Samples

This research was approved by the ethics committee at Kyushu University (Institutional Review Board (IRB) number 2019-232), and all patients participated with consent. This study included 172 patients who had surgical resection of pathological stage (pStage) I–III LUSC at our institution between January 2003 to December 2016. After surgery, patients were regularly checked through physical examinations, blood tests, and chest X-rays. They were monitored every 3 months for the first 2 years and then every 6 months afterward. Furthermore, patients underwent chest and abdominal computed tomography scans annually. If relapse was suspected, additional tests were conducted, including head magnetic resonance imaging and positron emission tomography scans. The recurrence date was determined as either the date of confirmed recurrence through histological examination or, in cases identified by clinical evidence, the date when the attending physician recognized the recurrent disease.

The clinical data and subsequent information were obtained from the patients' medical records. The pStage was redefined by the 8th edition of the TNM classification.[25]

Public Dataset

Information on 494 patients with LUSC, including clinical and genetic data, was obtained from The Cancer Genome Atlas (TCGA) database. Patients were divided into two groups based on median SIRPα mRNA expression, resulting in the high- and low-expression groups.

Immunohistochemistry (IHC)

IHC was attempted on formalin-fixed, paraffin-embedded tumor tissue segments of 4 μm thickness. Immunohistochemical staining for CD80, CD163, CD8, PD-L1, Foxp3, and Granzyme B was performed as previously described.[23,26–29] Briefly, the SIRPα and PD-1 staining procedure was as follows. The sections were treated with xylene and with a decreasing concentration of ethanol to remove the paraffin. Endogenous peroxidase activity was blocked by incubation for 30 min with 3% H_2O_2 in methanol. The samples were treated with TargetRetrieval Solution (Dako; pH 9.0 for SIRPα and PD-1) in a microwave oven at 100 ℃ for 15 min for SIRPα or a decloaking chamber at 121 ℃ for PD-1. Next, the sections were exposed to the primary Ab at 4 ℃ overnight. Bound Ab was identified using the DAKO EnVision Detection System manufactured by DakoCytomation. Finally, the sections were incubated with 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. We used samples from human tonsils or spleens as positive controls.

The IHC analysis was performed using Abs that are commercially available as follows: SIRPα (clone D613M, 1:100 dilution; Cell Signaling Technology), CD8 (clone 1A5, 1:100 dilution; BioGenex), CD80 (clone B7-1, 1:100 dilution; R&D Systems), CD163 (clone 10D6, 1:100 dilution; Leica), PD-L1(clone SP142, 1:00 dilution; Abcam), PD-1 (clone D4W2J, 1:100 dilution; Cell Signaling Technology), Foxp3 (clone 236A/E7, 1:100 dilution; Dako), and Granzyme B (1:100 dilution; abcam). Two experienced observers (K.T. and F.N.) who were unaware of the patient's clinical status reviewed all IHC data. SIRPα-positive tumor-infiltrating macrophages were measured by a high-powered field (HPF) with five fields of view and were subsequently

classified into two groups (high- and low-expression group) using the median of the total as the cutoff value. They were found in both the membrane and cytoplasm of cancer cells and cells infiltrating the tumor stroma. In this study, tumor cells were evaluated for PD-L1 expression on the membrane using the tumor proportion score (TPS). Cases with a TPS of ≥ 1% were classified as positive. Furthermore, the number of $CD8^+$, $CD80^+$, $CD163^+$, $PD\text{-}1^+$, $Foxp3^+$, and Granzyme B^+ cells were counted in five HPFs. Typical images of IHC staining for SIRPα, PD-L1, PD-1, CD8, CD80, CD163, Foxp3, and Granzyme B are shown in Fig. 1.

Cell Culture and Cytokine Treatment

We examined the expression of SIRPα in a human monocytic cell line (THP-1) and peripheral blood mononuclear cells (PBMC) from healthy donors. THP-1 was purchased from the Japanese Collection of Research Bioresources (JCRB). Permission to donate blood was obtained from the Ethics Committee of Kyushu University (IRB number 23173-00). In addition, we examined PD-L1 expression in two human lung squamous cell lines (EBC-1 and H520). EBC-1 was obtained from the JCRB and H520 was obtained from the American Type Culture Collection. The BD Vacutainer CPT tubes were used to extract PBMCs from the whole blood of healthy donors ($n = 3$), as directed by the manufacturer, using a density gradient centrifugation method. Monocytes from PBMCs were isolated using the EasySep® magnetic separation method according to the manufacturer's protocol. Multicolor flow cytometry confirmed that the isolated cells had a purity of over 90% in monocytes (Supplementary Fig. 1). All cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. All cell lines or monocytes were treated with 20 ng/mL recombinant interferon-gamma (IFNγ) or 50 ng/mL interleukin (IL)-2, IL-6, and tumor necrosis factor-alpha (TNFα) for 48 h before being collected for flow cytometry. All cytokines were HumanKine® from Proteintech.

Flow Cytometric Analysis

The cultured cells were stained using multicolor flow cytometry immediately after collection. To decrease nonspecific binding, single-cell suspensions ($0.5–1 \times 10^6$ cells) were prepared and incubated at room temperature for 10 min with an Fc receptor blocking solution and Brilliant dye buffer. Single-cell suspensions were subsequently treated with fluorescently labeled and isotype-labeled Abs specific to humans for 30 min at 4 °C, using multiple-color panels. Additional information about the two panels with multiple colors are presented in Supplementary Table 1. In short, the lymphocyte surface panel was created to verify the purity

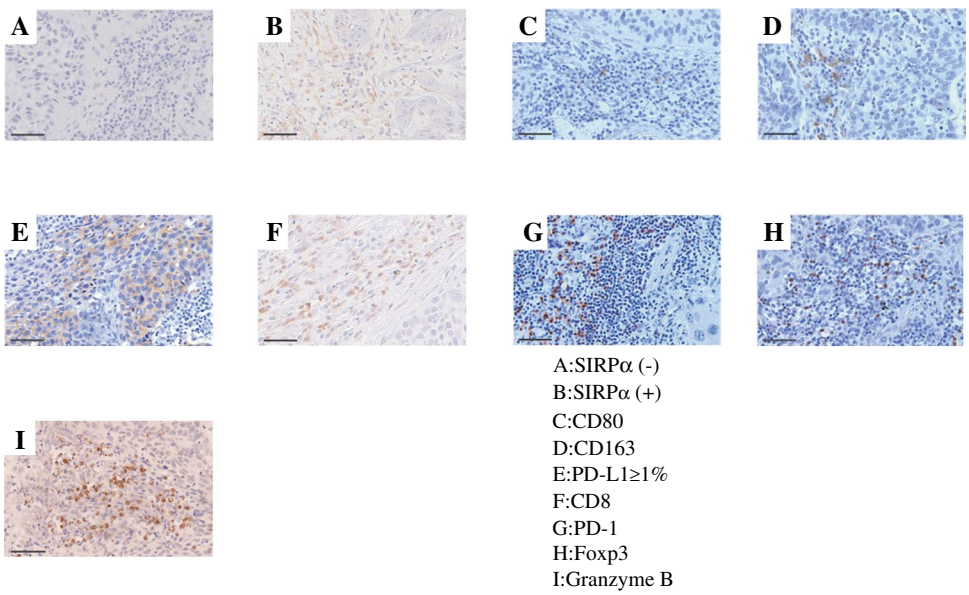

FIG. 1 Representative images of IHC staining of LUSC sections. Representative images of low SIRPα expression (**A**), high SIRPα expression (**B**), CD80 (**C**), and CD163 (**D**) in macrophages are shown. Representative image of cases with PD-L1 ≥ 1% are shown in (**E**). Representative images of CD8 (**F**), PD-1 (**G**), Foxp3 (**H**), Granzyme B (**I**) in tumor-infiltrating lymphocytes are shown. Scale bar, 50 μm. *CD8* cluster of differentiation 8, *CD80* cluster of differentiation 80, *CD163* cluster of differentiation 163, *Foxp3* Forkhead box protein P3, *IHC* immunohistochemistry, *LUSC* lung squamous cell carcinoma, *PD-1* programmed cell death 1, *PD-L1* programmed cell death-ligand 1, *SIRPα* signal-regulatory protein alpha

of monocytes. This panel included fluorescent dye-labeled anti-human antibodies against CD45, SIRPα, and CD14. The tumor cell surface panel was made to examine PD-L1 on tumor cells. This panel included fluorescent dye-labeled anti-human antibodies for EpCAM and PD-L1. Following the staining process, any extra antibody was removed by washing twice with phosphate-buffered saline (PBS). Single-cell suspensions were subsequently treated with 7-amino actinomycin D (7-AAD) for 5 min at room temperature to identify apoptotic cells. The analysis by flow cytometry was performed using the BD FACS Verse instrument (BD Biosciences, Franklin Lakes, NJ, USA). The data were processed using the FlowJo 10 software (Tree Star, Ashland, OR, USA) and displayed as histograms and mean fluorescence intensity (MFI). After gating single cells, monocytes gating $CD45^+$ cells were detected as $CD14^+$ cells; to analyze LUSC cells, gating was performed using tumor cell surface panels. After dead cells were removed and single cells gated, $EpCAM^+$ cells were gated.

Statistical Analysis

Fisher's exact test was employed to analyze relationships between categorical variables, while the sample *t*-test was used to examine relationships between continuous variables. Recurrence-free survival (RFS) was defined as the period during which the patient survived without cancer recurrence, and overall survival (OS) was defined as the period from surgery to death due to any cause. Patients with no events were censored at the last follow-up. The Kaplan–Meier method was used to derive survival curves using the log-rank test. Using the backward elimination method, a multivariate logistic regression model was used to calculate the odds ratio (OR) with a 95% confidence interval (CI) for SIRPα or SIRPα/PD-L1 co-expression associated with clinicopathological features. Survival data were analyzed using Cox proportional hazards regression analysis, and positive risk factor estimates were estimated using the backward elimination method to obtain hazard ratios (HRs). A Student's *t*-test was used for the analysis of in vitro data, and quantitative data were presented as mean ± standard deviation. All *P* values < 0.05 were considered statistically significant. JMP software v.16 and GraphPad Prism v10.1.1 were used for statistical analyses.

RESULTS

Association Between SIRPα Expression and Clinicopathological Characteristics

We examined 172 patients with pStage I–III LUSC. The clinicopathological features of the patients are presented in Supplementary Table 2. The median age of all patients was 71 years (range 45–87 years), 152 (88.4%) patients were male, and 162 (94.2%) had a history of smoking. There were 69 patients (40.1%) with pStage II or higher, and sublobar resection was selected as the surgical procedure in 38 patients (22.1%). Patients were divided into high- ($n = 86$) and low- ($n = 86$) expression groups following IHC staining for SIRPα. Supplementary Table 3 shows the association between SIRPα expression and clinicopathological factors. The high- and low-expression groups did not differ significantly in patient background information, such as age, gender, smoking history, pathological T factor, pathological N factor, pathological stage, pleural invasion, lymphovascular invasion, and surgical procedure.

Association Between SIRPα Expression on Tumor-Associated Macrophages and Tumor-Infiltrating Immune Cells or Tumor PD-L1 Expression

We aimed to determine whether SIRPα is associated with antitumor immunity in LUSC. For this purpose, the IHC analysis was employed to examine the relationship between SIRPα expression and M1 and M2 macrophages, CTLs, Tregs, and antitumor immune response activity. We used CD80, CD163, CD8, PD-1, Foxp3, and Granzyme B as markers of M1 and M2 macrophages, CTLs, immune checkpoint receptors expressed on CTLs, Tregs, and antitumor immune response activity, respectively. The median number of $CD80^+$ macrophages, $CD163^+$ macrophages, $CD8^+$ tumor-infiltrating lymphocytes (TILs), $PD\text{-}1^+$ TILs, $Foxp3^+$ TILs, and Granzyme B^+ TILs was 3.6 (range 1–8.8), 7.9 (range 1–18), 4.8 (range 2–23), 10 (range 2–40), 3 (range 0–12), and 12 (range 1–52), respectively. The high SIRPα expression group showed significantly more $CD163^+$ macrophages and $CD8^+$, $PD\text{-}1^+$, and $Foxp3^+$ cells compared with the low SIRPα expression group ($P = 0.0006$, $P = 0.0116$, $P = 0.0121$, and $P = 0.0422$, respectively; Fig. 2B–E). Conversely, there was no relationship between SIRPα expression and $CD80^+$ macrophages or Granzyme B^+ cells (Fig. 2A, F).

Next, we examined the relationship between SIRPα expression and expression of the immune checkpoint molecule PD-L1 in LUSC. In our cohort of patients with LUSC, 95 (58.6%) were PD-L1 positive (TPS ≥ 1%). The high SIRPα expression group included significantly more PD-L1-positive patients than the low SIRPα expression group ($P = 0.0139$, Supplementary Table 4). Furthermore, the multivariate analysis revealed a significant relationship between high SIRPα expression and increased levels of pathological infiltrating $CD163^+$ macrophages, $PD\text{-}1^+$ cells, and $PD\text{-}L1^+$ cells ($P = 0.0012$, $P = 0.0224$, and $P = 0.0406$, respectively; Table 1).

The relationship between the co-expression of SIRPα and PD-L1 and clinicopathological characteristics was investigated. On multivariate analysis, co-expression of SIRPα and

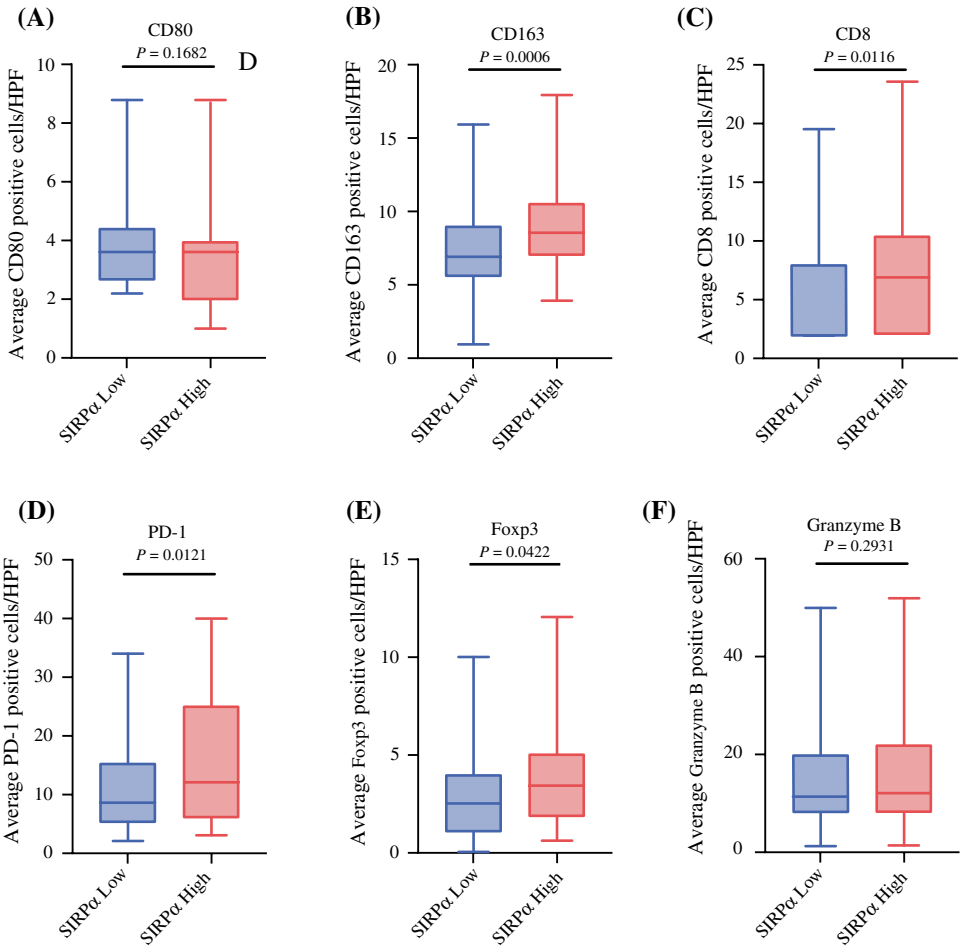

FIG. 2 The association between SIRPα expression and immune cells was evaluated by manually counting the number of cells in IHC sections. The association between tumor-associated macrophages and SIRPα expression was evaluated (**A**, **B**). $CD80^+$ macrophages did not correlate with SIRPα expression (**A**), while $CD163^+$ macrophages were significantly positively correlated with SIRPα expression (**B**). The association between TILs and SIRPα expression was assessed (**C**–**F**). CD8 (**C**), PD-1 (**D**), and Foxp3 (**E**) positively correlated with SIRPα expression significantly, while Granzyme B (**F**) did not. For each box plot, the top bar is the maximum observation, the lower bar is the minimum observation, the top of the box is the upper or third quartile, the bottom of the box is lower or first quartile, and the middle bar is the median value. *CD8* cluster of differentiation 8, *CD80* cluster of differentiation 80, *CD163* cluster of differentiation 163, *Foxp3* Forkhead box protein P3, *HPF* high-powered field, *IHC* immunohistochemistry, *ns* not significant, *PD-1* programmed cell death 1, *PD-L1* programmed cell death-ligand 1, *SIRPα* signal-regulatory protein alpha

PD-L1 was considerably correlated with $CD8^+$ cells, male sex, and pStage ≥ II ($P = 0.0003$, $P = 0.0344$, and $P = 0.0253$, respectively; Supplementary Table 5).

Impact of SIRPα Expression on RFS and OS

The median follow-up time was 4.2 years (range 0.0–18.3). The survival analyses showed that both RFS and OS had a significantly poorer prognosis in the high SIRPα expression group than in the low SIRPα expression group ($P = 0.0056$ and $P = 0.0439$, respectively, log-rank test; Fig. 3A, B). In this cohort, univariate and multivariable analyses were conducted to identify independent prognostic factors (Table 2). High SIRP expression was found to be one of the significant predictors of RFS and OS in the multivariate analysis ($P = 0.0042$ and $P = 0.0136$, respectively).

Impact of SIRPα mRNA Expression on OS in Patients with LUSC in the TCGA Dataset

We investigated the clinical impact of *SIRPα* expression on survival in patients with LUSC using the TCGA dataset. Initially, we evaluated SIRPα mRNA expression levels in 494 patients with LUSC from the TCGA dataset; the 494 patients were divided into high ($n = 247$) and low ($n = 247$) expression groups based on the level of SIRPα mRNA expression. The median follow-up was 1.8 years (range 0.0–14.5). The Kaplan–Meier curve revealed that

TABLE 1 Univariate and multivariate analyses of the relationship between high SIRPα expression and patients' clinicopathological characteristics

Characteristics		Univariate analysis			Multivariate analysis		
		OR	95% CI	*P* value	OR	95% CI	*P* value
Age	≥ 70/< 70	1.05	0.57–1.93	0.8769			
Sex	Male/Female	1.58	0.61–4.09	0.3443			
Smoking history	Smoker/Nonsmoker	0.65	0.18–2.39	0.5173			
pT	≥ T2/T1	1.22	0.66–2.27	0.5283			
pN	≥ N1/N0	0.94	0.48–1.86	0.8623			
pStage	≥ II/I	1.16	0.63–2.13	0.6408			
Pleural invasion	Yes/No	1.00	0.53–1.88	1.0000			
Lymphatic invasion	Yes/No	0.90	0.38–2.18	0.8228			
Vascular invasion	Yes/No	1.00	0.55–1.83	1.0000			
CD8[a]	Continuous variable	1.02	1.00–1.03	0.0105			
CD80[a]	Continuous variable	0.97	0.93–1.01	0.1640			
CD163[a]	Continuous variable	1.04	1.02–1.06	0.0005	1.04	1.01–1.06	0.0012
PD-1[a]	Continuous variable	1.04	1.01–1.07	0.0116	1.04	1.00–1.07	0.0224
PD-L1	Positive/Negative	2.25	1.22–4.16	0.0096	1.95	1.03–3.72	0.0406
Foxp3[a]	Continuous variable	1.03	1.00–1.06	0.0402			
Granzyme B[a]	Continuous variable	1.02	0.99–1.05	0.2890			

[a]Number of positive cells per high-power field

CD8 cluster of differentiation 8, *CD80* cluster of differentiation 80, *CD163* cluster of differentiation 163, *CI* confidence interval, *Foxp3* Forkhead box protein P3, *OR* odds ratio, *PD-1* programmed cell death 1, *PD-L1* programmed cell death-ligand 1, *pT* pathological T factor, *pN* pathological N factor, *pStage* pathological stage, *SIRPα* signal-regulatory protein alpha

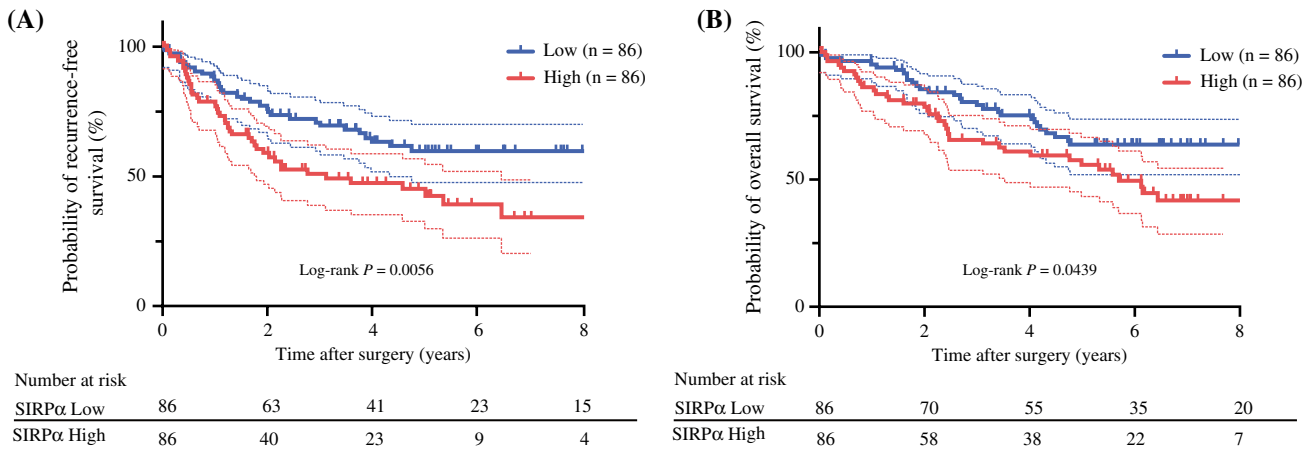

FIG. 3 Kaplan–Meier curves showing survival of patients with LUSC according to SIRPα expression. Recurrence-free survival (**A**) and overall survival (**B**) of the high ($n = 86$) and low ($n = 86$) SIRPα expression groups. Statistical analysis was undertaken using the log-rank test. *LUSC* lung squamous cell carcinoma, *SIRPα* signal-regulatory protein alpha

the high group had a significantly lower OS than the low group (Supplementary Fig. 2). Univariate and multivariate analyses were performed to identify independent prognostic factors in this patient cohort (Supplementary Table 6), and high expression of SIRPα was one of the significant prognostic factors for OS on multivariate analysis ($P = 0.0034$).

SIRPα Expression in the Monocyte Cell Line

To investigate the factors responsible for the increased expression of SIRPα, THP-1 and healthy human monocytes were evaluated by flow cytometry. When comparing untreated control cells with cytokine-treated cells, IFNγ

TABLE 2 Univariate and multivariate analyses of recurrence-free survival and overall survival

Characteristics		Recurrence-free survival						Overall survival					
		Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
		HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	≥ 70/< 70	1.78	1.08–2.93	0.0225	2.13	1.29–3.52	0.0032	1.96	1.17–3.28	0.0104	2.68	1.58–4.53	0.0002
Sex	Male/Female	1.29	0.59–2.80	0.5274				1.13	0.52–2.47	0.7536			
Smoking history	Smoker/Nonsmoker	0.67	0.29–1.55	0.3512				0.76	0.30–1.90	0.5624			
pT	≥ T2/T1	1.01	0.63–1.63	0.9536				1.08	0.66–1.76	0.7628			
pN	≥ N1/N0	2.52	1.58–4.02	0.0001				1.53	0.92–2.51	0.0981			
pStage	≥ II/I	2.46	1.56–3.89	0.0001	2.33	1.43–3.78	0.0006	1.91	1.20–3.06	0.0067	1.85	1.13–3.03	0.0138
Pleural invasion	Yes/No	1.54	0.97–2.45	0.0679				1.60	0.99–2.58	0.0566			
Lymphatic invasion	Yes/No	3.48	2.06–5.88	< 0.0001	3.22	1.86–5.60	< 0.0001	2.27	1.28–4.03	0.0052	2.04	1.11–3.74	0.0208
Vascular invasion	Yes/No	1.82	1.16–2.87	0.0098				1.34	0.84–2.15	0.2194			
Surgical procedure	Sublober resection/≥ Lobectomy	1.17	0.69–1.99	0.5592				1.49	0.87–2.55	0.1470			
CD8[a]	Continuous variable	0.98	0.94–1.02	0.4151				1.00	0.99–1.01	0.4812			
CD80[a]	Continuous variable	0.87	0.71–1.04	0.1315				0.90	0.73–1.08	0.2624			
CD163[a]	Continuous variable	1.03	0.95–1.11	0.5021				1.02	0.93–1.10	0.7205			
PD-1[a]	Continuous variable	1.02	1.00–1.04	0.0610				1.03	1.00–1.05	0.0079	1.04	1.01–1.06	0.0094
PD-L1 expression	Positive/Negative	1.15	0.73–1.81	0.5575				1.09	0.68–1.75	0.7177			
Foxp3[a]	Continuous variable	0.91	0.82–1.00	0.0901				0.91	0.81–1.01	0.0936	0.85	0.74–0.96	0.0022
Granzyme B[a]	Continuous variable	0.99	0.97–1.02	0.6293				0.99	0.96–1.02	0.4958			
SIRPα expression	High/Low	1.90	1.20–3.00	0.0064	1.98	1.24–3.17	0.0042	1.62	1.00–2.60	0.0459	1.89	1.14–3.14	0.0136

[a]Number of positive cells/high-power field

CD8 cluster of differentiation 8, *CD80* cluster of differentiation 80, *CD163* cluster of differentiation 163, *CI* confidence interval, *Foxp3* Forkhead box protein P3, *HR* hazard ratio, *PD-1* programmed cell death 1, *PD-L1* programmed cell death-ligand 1, *pT* pathological T factor, *pN* pathological N factor, *pStage* pathological stage, *SIRPα* signal-regulatory protein alpha

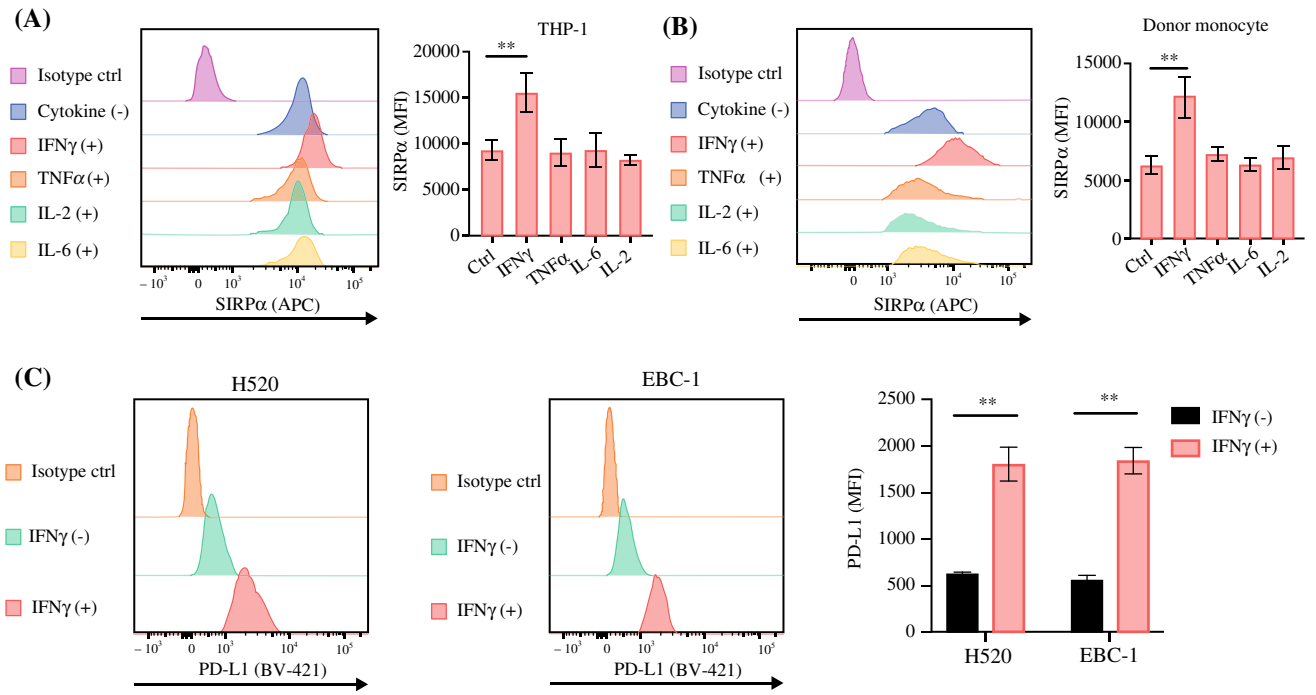

FIG. 4 SIRPα expression on monocytes and PD-L1 expression on LUSC cell lines are upregulated by IFNγ. **A** SIRPα expression on THP-1 cell lines that were treated with cytokines for 48 h. **B** SIRPα expression on monocytes from healthy donors that were treated with cytokines for 48 h. **C** PD-L1 expression on LUSC cell lines (H520 and EBC-1) that were treated with IFNγ for 48 h. SIRPα and PD-L1 expression were measured by multicolor flow cytometry. The data are represented by histograms and MFI values. $^{**}P < 0.01$. *IFNγ* interferon-gamma, *IL-2* interleukin-2, *IL-6* interleukin-6, *LUSC* lung squamous cell carcinoma, *MFI* mean fluorescence intensity, *PD-L1* programmed cell death-ligand 1, *SIRPα* signal-regulatory protein alpha, *TNFα* tumor necrosis factor-alpha

increased the expression of SIRPα to significantly higher levels than control (Fig. 4A, B).

Effect of IFNγ on PD-L1 Expression in Human Squamous Cell Carcinoma Cell Lines

To examine the effect of IFNγ on PD-L1 expression in human squamous cell carcinoma cell lines, cells were treated with IFNγ (20 ng/mL) followed by flow cytometry evaluation. In two cell lines (H520 and EBC-1), IFNγ significantly increased PD-L1 expression compared with the control (Fig. 4C).

DISCUSSION

SIRPα binds to CD47, which triggers a signaling cascade that inhibits phagocytosis of target cells and functions when inversely linked to antitumor.[20] In the present study, we emphasized the prognostic impact of SIRPα expression in LUSC.

We examined the correlation between SIRPα expression and clinicopathological features, such as tumor-associated macrophages (TAMs), PD-L1 expression, and $CD8^+$, PD-1, $Foxp3^+$, and Granzyme B^+ TILs, using samples from resected LUSC samples, in addition to its role as a prognostic factor. SIRPα expression did not correlate with patient background, such as stage, tumor factors, or lymph node metastasis factors. In the multivariate analysis, $CD163^+$ macrophages, $PD\text{-}1^+$ TILs, and PD-L1 expression were independent predictors of high SIRPα expression. In addition, high SIRPα expression was an independent poor prognostic factor for RFS and OS. Furthermore, the in silico analysis revealed that the high SIRPα expression group had a poor prognosis. Although a report has shown that a population with high SIRPα/CD68 in the TME of NSCLC has a poor prognosis, there are no previous reports on the significance of SIRPα expression in LUSC and assessing TILs, including PD-L1, which may be clinically important.[30]

We showed that SIRPα expression significantly correlated with $CD163^+$ macrophage infiltration in LUSC, suggesting that $CD163^+$ macrophages may express high levels of SIRPα. Previous reports have shown that SIRPα correlates with M2 macrophages, which is consistent with the present results.[23,24] In malignancy, macrophages fall into two categories: M1 macrophages are included in T-helper 1 cell responses to pathogens and promote antitumor immunity, while M2 macrophages are included in T-helper 2 cell responses and suppress antitumor immunity.[31,32] Previous

studies have shown that TAMs, consisting primarily of M2 macrophages, convert the tumor microenvironment (TME) into an immunosuppressive and tumor-progressive state, leading to poor prognosis.[32] These findings indicate that SIRPα could suppress antitumor immunity via TAMs. On the other hand, SIRPα is considered to play a significant role in macrophage polarity; however, the mechanism remains unknown.[33,34]

Interestingly, this study also revealed that SIRPα expression in LUSC was strongly associated with PD-L1 expression. This result indicated that SIRPα expression might signal a "hot" tumor environment. The most common mechanisms for elevated PD-L1 expression are intrinsic and extrinsic induction.[35] Intrinsic induction refers to the upregulation of PD-L1 expression by stem cell signaling, genomic aberrations, epigenetic alterations, or constitutive oncogenic signaling.[36] On the other hand, extrinsic induction is the upregulation of PD-L1 expression by exogenous factors such as inflammatory cytokines.[37,38] We hypothesized that the correlation between PD-L1 and SIRPα expression may be due to a common factor, which is extrinsic induction. In vitro, SIRPα expression was upregulated by the inflammatory signature protein IFNγ. PD-L1 is also upregulated by IFNγ, as shown in previous reports and the present experiments.[39,40] The upstream pathway in SIRPα is not well understood; however, reports have suggested that inflammatory cytokines upregulate SIRPα in macrophages via signal transducer and activator of transcription (STAT) 3.[41] IFNγ activates the JAK/STAT pathway and upregulates STAT1. SIRPα proteins may share an upstream pathway with PD-L1, which is upregulated via STAT1/3; however, this requires further validation.[42] IFNγ activates antitumor immunity, such as T cells, but also suppresses antitumor immunity by upregulating PD-L1 expression in tumors.[37,38] In addition, IFNγ promotes SIRPα expression, which suppresses the innate immune system under the condition of hot tumors. In this study, SIRPα/PD-L1 co-expression was significantly associated with a high density of $CD8^+$ T cells. This suggests that the expression of both PD-L1 and SIRPα was induced by IFNγ released from $CD8^+$ T cells. SIRPα expression was also associated with $PD\text{-}1^+$ and $Foxp3^+$ cells. The correlation with PD-1, a marker of T-cell fatigue, may indicate that patients are not responding to antitumor immunity despite their hot tumor status, and are in a phase of immune escape.[43,44] Tregs also suppress the maturation of antigen-presenting cell and the activation of cytotoxic T cells. These findings indicate that an increase in SIRPα expression may induce a poor prognosis.

Numerous studies on blocking the CD47/SIRPα pathway have been reported.[22,45–47] Antitumor effects have been observed in many preclinical studies for anti-CD47 and anti-SIRPα antibodies against solid tumors and hematopoietic cancers. Several clinical trials are underway to investigate the effectiveness of CD47/SIRPα-targeted inhibitors.[48,49] According to the current study, the situation of high SIRPα expression is a hot tumor condition, which may respond well to anti-PD-L1 and anti-PD-1 antibodies. This is clinically important and should be studied in the future as a potential biomarker for forecasting the effectiveness of LUSC immunotherapy. Indeed, SIRPα expression has been reported to correlate with immune checkpoint inhibitor reactivity in melanoma.[50] The use of anti-SIRPα antibodies in hot tumors that are in a state of immune escape can increase cancer antigen presentation and turn the cancer immune cycle around.[51] In other words, treatment with the combination of CD47/SIRPα and PD-1/PD-L1 inhibitors may enhance the prognosis of patients with LUSC by reactivating both the innate and adaptive immune responses associated with macrophages and T cells, respectively. Several preclinical studies have demonstrated synergistic antitumor effects in mouse models of colon cancer and melanoma by blocking both CD47/SIRPα and PD-1/PD-L1 axes.[22]

This study has several limitations. First, this was a retrospective observational study performed at a single institution. Further confirmation of the present results may require a validation study in a larger cohort. Second, there are no clear guidelines for using or quantifying antibodies for SIRPα expression in NSCLC, and positive cutoff values vary from report to report. Therefore, further examination is necessary regarding the IHC evaluation of SIRPα as well as validation of the evaluation method used in our study. Third, our evaluation of SIRPα expression was limited to patients with surgically resected LUSC. Analysis of SIRPα expression in patients with advanced, unresectable, or recurrent disease may provide insight into its therapeutic potential. Fourth, the observation period was from 2003, and the treatment regimen after recurrence was different. The development of drug therapy for lung cancer has been remarkable in recent years, and it is highly possible that the timing of recurrence affected the prognosis.

In conclusion, SIRPα expression significantly predicts poor prognosis in patients with surgically resected LUSC. It is expected to be a target for combination therapy with anti-PD-1/L1 antibodies.

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