in vivo *38*: 474-481 (2024)
doi:10.21873/invivo.13462

Association Between Clinicopathological Parameters and S100A8/A9 Expression According to Smoking History in Patients With Non-small Cell Lung Cancer

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Abstract. *Background/Aim: Lung cancer is a major cause of cancer-related deaths worldwide, and chronic inflammation caused by cigarette smoke plays a crucial role in the development and progression of this disease. S100A8/9 and RAGE are associated with chronic inflammatory diseases and cancer. This study aimed to investigate the expression of S100A8/9, HMBG1, and other related pro-inflammatory molecules and clinical characteristics in patients with non-small cell lung cancer (NSCLC). Patients and Methods: We obtained serum and bronchoalveolar lavage (BAL) fluid samples from 107 patients and categorized them as never or ever-smokers. We measured the levels of S100A8/9, RAGE, and HMGB1 in the collected samples using enzyme-linked immunosorbent kits. Immunohistochemical staining was also performed to assess the expression of S100A8/9, CD11b, and CD8 in lung cancer tissues. The correlation between the expression of these proteins and the clinical characteristics of patients with NSCLC was also explored. Results: The expression of S100A8/A9, RAGE, and HMGB was significantly correlated with smoking status and was higher in people with a history of smoking or who were currently smoking. There was a positive correlation between serum and BAL fluid S100A8/9 levels. The expression of S100A8/A9 and CD8 in lung tumor tissues was significantly correlated with smoking history in patients with NSCLC. Ever-smokers, non-adenocarcinoma histology, and high PD-L1 expression were significant factors predicting high serum S100A8/9 levels in multivariate analysis. Conclusion: The S100A8/9-RAGE pathway and CD8 expression were increased in smoking-related NSCLC patients. The S100A8/9-RAGE pathway could be a promising biomarker for chronic airway inflammation and carcinogenesis in smoking-related lung diseases.*

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Key Words: Lung cancer, smoke, inflammation, S100A8/A9, RAGE.

Lung cancer remains a leading cause of neoplasm-related mortality worldwide. Smoking is a preventable risk factor for lung cancer, and it causes over 80% of lung cancer cases (1). Cigarette smoke (CS)-induced chronic inflammation is an important contributor to both the development and progression of lung cancer (2). CS-induced chronic inflammation accumulates pro-inflammatory cytokines, including transforming growth factor (TGF)-β, epidermal growth factor receptor (EGFR), interleukin (IL)-1, IL-8, and granulocyte colony-stimulating factor (G-CSF), through the oxidative stress pathway (2). Molecular signaling of this inflammatory pathway is believed to play a key role in cancer development and progression, and conversely, cancer causes chronic inflammation (3). Decades of cancer research have focused on potential targets for preventing lung carcinogenesis through epidemiologic studies, clinical trials, and preclinical investigations (4).

S100A8 and S100A9, the S100 protein family is consisted of 25 homologous low-molecular-weight intracellular calcium-binding proteins and have a role in damage-associated molecular patterns (DAMPs) in promoting immune response and repair mechanism during inflammation and tumor (5). The extracellular functions of S100 proteins are associated with chronic inflammatory diseases and

carcinogenesis (6). The receptor for advanced glycation end-products (RAGE) is a multiple-ligand receptor of the immunoglobulin superfamily (7). RAGE is a multiligand receptor, and its ligands include high mobility group box 1 (HMGB1), S100A8/A9, and some AGE species during cellular or physiologic stress (8). The activated RAGE signaling pathway has an important role in inflammatory reactions, including the activation of NF-κB, MAPKs, PI3K/Akt, Rho GTPases, Jak/STAT, and Src family kinases (8). CS is a common environmental risk factor in airway inflammation and upregulates the RAGE ligand (9).

S100A8 and S100A9 have been shown to be overexpressed in various kinds of malignancies and are associated with the carcinogenesis and metastatic processes. S100A8 and S100A9 upregulation inhibited the differentiation to dendritic cells (DCs) and accumulated myeloid-derived suppressor cells (MDSCs), supporting immune-response modulation and promoting tumor progression (10, 11). Previous studies showed that RAGE knockout enhanced CS-induced airway inflammation by downregulating S100A8/A9 and related inflammation and immune responses (12). Mice lacking S100A9 showed decreased accumulations of MDSCs (13). S100A9 expression in lung cancer patients was increased in ever-smokers compared to never-smokers (14). However, data exploring S100A8/A9-RAGE signals in CS-induced airway inflammation and patients' clinical features are limited. We previously revealed that the S100A8/A9-RAGE pathway was the mainstream of inflammation in CS-induced lung carcinogenesis (15). However, assessments of patients' clinical features have not yet been completed. In the present study, we aimed to investigate the expression of S100A8, S100A9, HMBG1, and other related pro-inflammatory molecules and the clinical characteristics of patients with non-small cell lung cancer.

Patients and Methods

Study population. We consecutively collected serum and bronchoalveolar lavage (BAL) fluid samples from 107 patients with pathologically confirmed lung cancer from January 2021 to December 2022 at The Catholic University of Eunpyeong St. Mary's Hospital, Korea. The following data were extracted from the medical records: patient demographics, smoking history, lung cancer stage, histologic type, and clinical outcomes of immune checkpoint inhibitors. The patients were categorized as never-smokers (<100 cigarettes in their life-time/zero cigarettes lifelong) or ever-smokers (former and current). Histological classification of tumors was based on World Health Organization criteria, and qualified pathologists evaluated the lung specimens. Tumor stages were classified according to the TNM Classification of Malignant Tumors, and tumor type was recorded at the time of diagnosis. *EGFR* mutations were examined using a PNA Clamp™ EGFR Mutation Detection Kit (PANAGENE, Inc., Daejeon, Republic of Korea) and real-time polymerase chain reaction (PCR) analysis. PD-L1 immunohistochemistry was performed using a VENTANA PDL1 (SP263) assay. PD-L1 expression was subclassified as high PD-L1 expression (TPS ≥50%) and low PD-L1 expression (TPS <50%). Cancer progression was defined as cancer that progressed radiographically after treatment. Progression was evaluated by the treating pulmonologists and independent radiologists. Progression-free survival (PFS) was defined as the time (month) from the date of initiation of ICI treatment to the date of progression. This study was approved by the Institutional Review Board of The Catholic University of Korea (PC22SISI0192).

Serum and bronchoalveolar lavage fluid. Blood samples collected in sodium heparin vacutainer tubes were mixed gently, centrifuged at 1,540×g at room temperature for 20 min, and serum was collected and immediately frozen. BAL fluid (BALF) was obtained at the site of lung infiltration using a flexible bronchoscope by experienced pulmonologists. For study sampling, 50-100 cc of normal saline was instilled, and at least 30% of BALF was withdrawn. The BALF was immediately filtered using a 0.45-μm filter syringe to remove debris and then concentrated using an Amicon Ultra-15 centrifugal filter (Millipore, MA, USA). Both samples were stored at –80˚C until analysis, at which time the samples were thawed at room temperature and placed on ice.

Enzyme-linked immunosorbent assay and cytokine assay. The concentration of S100A8/9, RAGE, and HMBG1 (MyBioSource, Inc. San Diego, CA, USA or Arigobio, Hsinchu, Taiwan, ROC) were measured in human serum or BALF using enzyme-linked immunosorbent (ELISA) kits. All protocols were performed according to the manufacturer's instructions. The minimum detectable concentration of S100A8/9 and RAGE was 0.625 ng/ml and 1.23 pg/ml, respectively, and 15.9 pg/ml in serum and 0.3 ng/ml in BALF for HMBG1.

Immunohistochemistry. After identifying samples in the medical records, adequate formalin-fixed, paraffin-embedded tissue blocks were obtained from the Department of Pathology. Tissues section at 4 μm thickness were prepared for immunohistochemical (IHC) staining and subsequently deparaffinized. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Antigens were retrieved by boiling in sodium citrate buffer (pH 6.0) for 15 min in the microwave. After reducing nonspecific background staining with 1.5% normal horse serum, the slides were incubated with primary monoclonal rabbit anti-human S100A8, S100A9, CD11b, and CD8 antibodies at 4˚C overnight and then incubated with biotin-labeled secondary antibodies. The immunoreactive signal was visualized with liquid 3,30-diaminobenzidine (DAB) in a buffered substrate (Dakocytomation, Carpinteria, CA, USA) and counter-stained with hematoxylin. Normal horse serum was used for the negative controls, and the primary antibodies were omitted. IHC expression was quantitated in the five most representative high-magnification fields (400×) randomly selected per tissue section, and at least 500 cells were counted per field in a semiquantitative scoring method. Two independent individuals who were unaware of patient information and the objective of the study examined the slides and corresponding tissue samples using a microscope. The presence of brown-yellow granules in the cytoplasm, nucleus, or cell membrane of the tumor cells was considered indicative of positive staining. Positivity in the slices was scored as follows: 0 for less than 5% positive cells, 1 for 5-25% positive cells, 2 for 25-50% positive cells, 3 for 50-75% positive cells, and 4 for more than 75% positive cells.

Defining cutoff values. A receiver operating characteristic (ROC) curve was generated for serum S100A8/9 to determine the cutoff value for smoking history with optimal sensitivity and specificity. The patients were then allocated to high or low groups based on the cutoff value.

Data analyses. All data are expressed as means±standard deviation. The statistically significant differences between the never- and ever-smoker groups were analyzed using the Kruskal Wallis test or the Mann Whitney *U*-test for independent samples. Correlations in univariate and multivariate logistic regression analyses were used to determine the clinicopathologic factors associated with high serum S100A8/9 levels. Pearson's correlation coefficient was used for correlation analysis between serum and BAL S100A8/9 levels. Statistical analysis was carried out using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA), with a p-value <0.05 considered statistically significant.

Results

Patient characteristics. A total of 107 patients were evaluated in this study. The patients' ages ranged from 45 to 88 years, with a mean age of 65.3 years. Out of 107 patients, 74 (69.2%) were male, and 33 (30.8%) were female. Among them, 28 patients (26.2%) had no history of smoking, while the remaining 79 patients (73.8%) were either ex- or current smokers. Regarding the pathology type, 66 patients (61.7%) had adenocarcinoma, 31 (29.0%) had squamous cell carcinoma, and the remaining 10 patients (9.3%) had other types. The initial lung cancer stage at diagnosis was categorized into stages I-IV. In this study, 36 patients (33.6%) were classified as having high PD-L1 expression, whereas 71 patients (66.4%) were classified as low. The median PFS in stage III-IV patients treated with ICIs, such as pembrolizumab, nivolumab, and atezolizumab, was 5.4 months (Table I). A total of 107 patients were divided into high and low S100A8/9 groups based on the cutoff level from the ROC curve. The area under the ROC curve for serum S100A8/9 was 0.704, and the p-value was statistically significant at <0.004. We set a cutoff level of 450 ng/ml, which was the high sensitivity and specificity point for RES.

Univariate and multivariate analyses of serum and BALF. The expression of S100A8/9 and HMGB-1 was higher in BALF than in serum, whereas RAGE showed similar values in both samples (Table I). Regarding the average differences in S100A8/A9, RAGE, and HMGB levels based on smoking (never *vs*. ever), Mann-Whitney *U*-test was conducted. Both serum and BALF levels were significantly higher in the ever-smoked group compared to the never-smoked group (p<0.05 each) (Figure 1). Pearson's correlation analysis showed a positive correlation between serum and BALF S100A8/9 levels. The scatter plot for serum and BAL/f S100A8/9 levels is presented in Figure 2. Additionally, in terms of the type of lung cancer, adenocarcinoma showed higher S100A8/A9 expression compared to squamous cell carcinoma (p=0.009).

Table I. *Clinical information summary and measurement of inflammatory markers in study participants with lung cancer.*

Parameters	Current smoker (n=107)
Age, mean	70.4±9.5
Sex	
Male	74 (69.2%)
Female	33 (30.8%)
Smoking history	
Never	28 (26.2%)
Ever	79 (73.8%)
Pathology	
Adenocarcinoma	66 (61.7%)
Squamous	31 (29.0%)
Others	10 (9.3%)
Initial stage	
I	20 (18.7%)
II	21 (19.6%)
III	27 (25.2%)
IV	39 (36.4%)
EGFR mutation	29 (27.1%)
PD-L1 TPS (SP263)	
<49%	71 (66.4%)
≥50%	36 (33.6%)
PFS, median, IQR (months)	5.4
S100A8/9 (serum) ng/ml	466.2±213.4
S100A8/9 (BALF) ng/ml	1,428.7±355.6
RAGE (serum) pg/ml	562.6±232.3
RAGE (BALF) pg/ml	435.4±494.9
HMGB-1 (serum) pg/ml	52.1±33.6
HMGB-1 (BALF) ng/ml	0.2±0.1

PFS: Progression-free survival; IQR: interquartile range; EGFR: epidermal growth factor receptor; PD-L1 TPS: programmed death ligand-1 tumor proportion score.

No significant difference was observed based on clinical staging (p=0.288) or sex (p=0.175) (data not shown). The results of the logistic regression analysis identifying the clinicopathologic factors associated with high serum S100A8/9 levels are presented in Table II. Male sex [hazard ratio (HR)=0.175, 95% confidence interval (CI)=0.058–0.528, p=0.002], ever-smoked (HR=6.731, 95%CI=2.053–22.067, p=0.002), non-adenocarcinoma histology (HR=0.778, 95%CI=0.319–1.896, p=0.058), wild-type *EGFR* (HR=0.341, 95%CI=0.118–0.985, p=0.047), and high PD-L1 expression ≥50% (HR=4.200, 95%CI=1.638–10.772, p=0.003) had p-values of less than 0.2 in the univariate analysis. Ever-smoked, non-adenocarcinoma histology, and high PD-L1 expression remained statistically significant in multivariate analysis.

Immunohistochemical expression. The staining of lung tumor tissues was generally heterogeneous, with some tumors showing rare positively stained tumor cells, some showing scattered positively stained cells, and some showing uniform staining, with the majority of tumor cells stained positively for

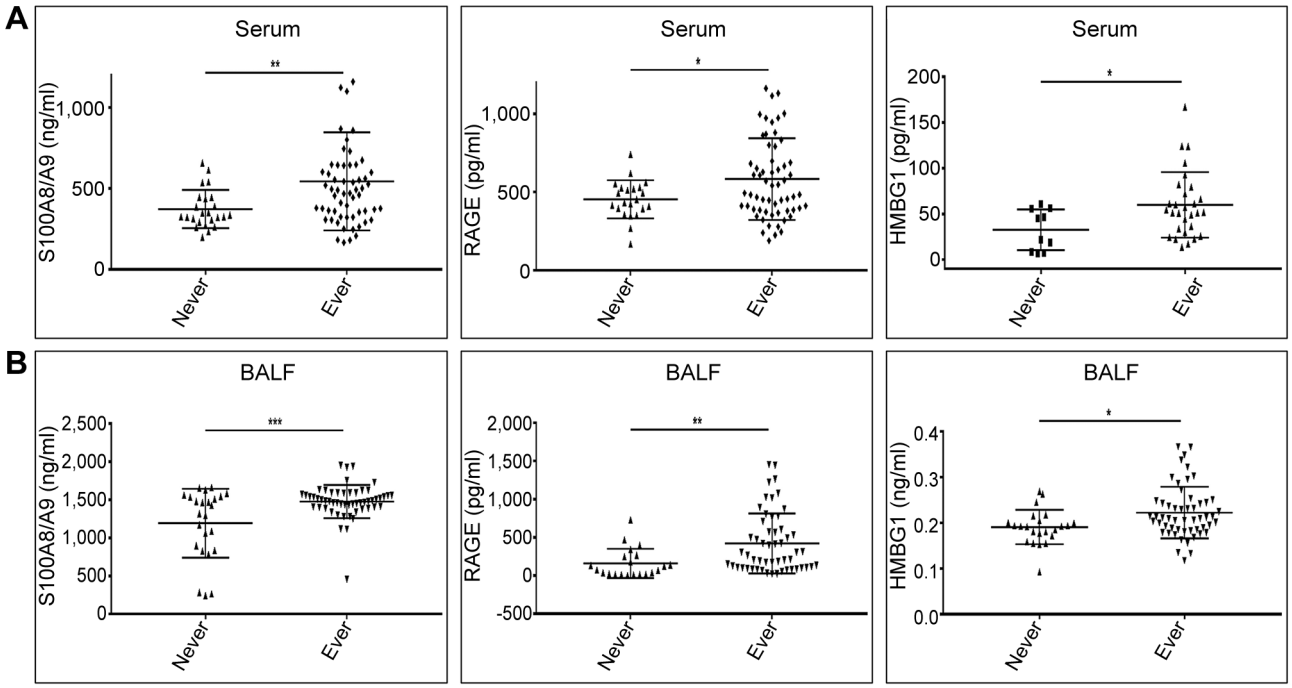

Figure 1. *The expression of S100A8/A9-RAGE pathway-related genes in human lung cancer patients. S100A8/9, RAGE, and HMBG1 levels were analyzed in (A) serum and (B) BALF by ELISA assays. The results are presented as the mean (±SD). Significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001 compared to the never-smoker group.*

S100A8 and S100A9. S100A8 and S100A9 were expressed in the cytoplasm and nucleus of adenocarcinoma cells and the extracellular matrix in the vicinity of inflammatory cells, mainly located in the cytoplasm, stained with brown particles, and distributed evenly. Smokers had higher S100A8 and S100A9 expression, whereas there was no significant correlation between S100A8 and S100A9 immunostaining and patient age, sex, or degree of differentiation. The positive expression scores for S100A8 and S100A9 in the smoking group were higher than those in the never-smoked group, with statistical significance (Figure 3A). To examine the relative abundance of CD8 and CD11b (macrophage marker) in human lung cancer, we also compared the proportion of these cells in adenocarcinoma lung tissue sections by IHC and analyzed the results based on smoking history and clinical staging. Individuals with a smoking history appeared to have a higher expression of CD8 (p<0.001) compared to never-smokers, whereas CD11b showed a non-significant but increasing trend in the smoking group (Figure 3B). However, no significant differences were observed in the expression of CD8 and CD11b based on clinical staging.

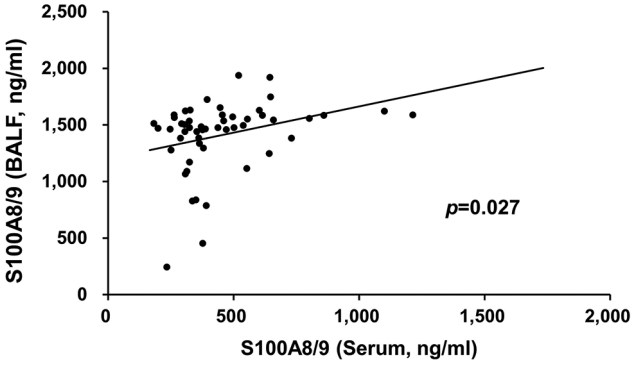

Figure 2. *Correlation of S100A8/9 levels between serum and BAL in patients with non-small cell lung cancer.*

Discussion

In this study, we detected the expression of S100A8/9, RAGE, and HMGB1 proteins in the serum and BALF of 107 NSCLC cancer patients and explored the correlation between the expression of these proteins and the clinical characteristics of NSCLC patients, such as age, sex, pathological type, clinical stage and, especially, smoking status. The expression of S100A8/A9, RAGE, and HMGB, as well as CD8, was significantly correlated with smoking status and was higher in people with a history of smoking or who were currently smoking. In addition, although the data are not shown in the main text, the levels of these factors in

Table II. *Univariate and multivariate analyses for predicting high serum S100A8/9 levels in patients with non-small cell lung cancer.*

		Univariate		Multivariate	
Parameters	No	HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
Sex	Female *vs*. male (Ref)	0.175 (0.058-0.528)	0.002		
Age		1.000 (0.957-1.044)	0.996		
Initial stage	III-IV *vs*. I-II (Ref)	1.257 (0.522-3.026)	0.610		
Smoking status	Ever *vs*. never (Ref)	6.731 (2.053-22.067)	0.002	5.994 (1.092-32.905)	0.039
Pathology	Others *vs*. adenocarcinoma (Ref)	0.778 (0.319-1.896)	0.058	0.184 (0.053-0.633)	0.007
EGFR	Mutation *vs*. wild-type (Ref)	0.341 (0.118-0.985)	0.047		
PD-L1 (SP263)	≥50% *vs*. <50% (Ref)	4.200 (1.638-10.7772)	0.003	7.672 (2.236-26.326)	0.001

the serum and BALF samples of normal people were similar to those of never-smokers.

Smoking damages the respiratory tract and can cause chronic inflammation of the airways (16). This inflammation can lead to chronic obstructive pulmonary disease (COPD), pneumonia, and other respiratory diseases (17). Chronic inflammation can also promote the development of cancer by causing DNA damage and by creating a tumor microenvironment that promotes the growth and spread of cancer cells (18). Most lung cancer research has been done on advanced-stage lung cancer, and little research has been conducted on the immune evasion mechanisms and novel biomarkers associated with smoking in early-stage patients and high-risk groups. Basic research has mainly been performed using lung damage models or normal epithelial cells, and little has been done on the role of inflammatory mediators in the actual occurrence and progression of cancer cells and the effect of their blockade. A previous study by our research team found activated immune mediators, including the S100A8/A9-RAGE signaling pathway, in lung cells exposed to tobacco smoke extract (15). In the future, S100A8/9 protein might be applied as a therapeutic target in the clinical treatment of lung cancer in the era of immuno-oncology (19).

Cancer immunoediting consists of three phases (elimination, equilibrium, and escape), and an immunosuppressive tumor microenvironment may lead to escape, where cancer becomes a clinically apparent disease (20). Chronic inflammation can promote tumor growth and metastasis by inducing an immune escape mechanism that allows cancer cells to escape from the normal immune system. Macrophages, regulatory T and B cells, and MDSCs are all known to play a role in this process (21, 22). In this study, we used immunohistochemical methods to detect the expression of S100A8 and S100A9 proteins, as well as CD8 and CD11b, in the tissue of NSCLC cancer patients and confirmed the expression of inflammatory and immune factors. CD11b is involved in the diverse adhesive interactions of monocytes, macrophages, and granulocytes (23). In contrast, CD8 is a transmembrane glycoprotein that functions as a co-receptor alongside the T-cell receptor (TCR). Together with the TCR, the CD8 co-receptor contributes to T cell signaling and facilitates cytotoxic T cell-antigen interactions (24). The potential role of CD8-expressing cells and their therapeutic implication in smoking-induced lung cancer should be further elucidated.

This study did not find a correlation between S100A8 and S100A9 and patient age, sex, or clinical stage, as in the study published by Su *et al*. (25). However, S100A8 and S100A9, and their related factors RAGE and HMGB1, were significantly different between never-smokers and ever-smokers. In addition, the levels of S100A8/A9 in serum and BALF were positively correlated, suggesting that serum could be a promising biomarker in patients with smoking-related lung cancer. The expression of PD-L1 was significantly higher in patients with higher S100A8 and S100A9 levels, indicating that these factors could affect the clinical outcome of patients with lung cancer who received ICI therapy.

MDSCs are a major component of the immunosuppressive tumor microenvironment and have been recognized as a factor contributing to inflammation-related cancers (26). Ortiz *et al*. exposed mice to CS and showed MDSC accumulation with potent immunosuppressive activity in various organs, and development of tumor lesion (13). S100A8/A9 regulated the MDSC-mediated immunosuppressive tumor microenvironment by RAGE and the Toll-like receptor (TLR)4 signaling pathway in colorectal carcinoma (27). In lung cancer patients with COPD, COPD severity was positively correlated with the co-expression of PD-l/TIM-3 in CD8 T cells (28). We could hypothesize that smoking-related inflammation affected higher tumor-infiltrating lymphocyte exhaustion and had an impaired protective impact on immune cells in COPD patients, which is supported by previous reports demonstrating that smoking was associated with immune exhaustion (PD-1, TIM-3, and CTLA4) (29).

Although S100A8/A9 protein contributes to many types of inflammation-associated diseases, ongoing studies will

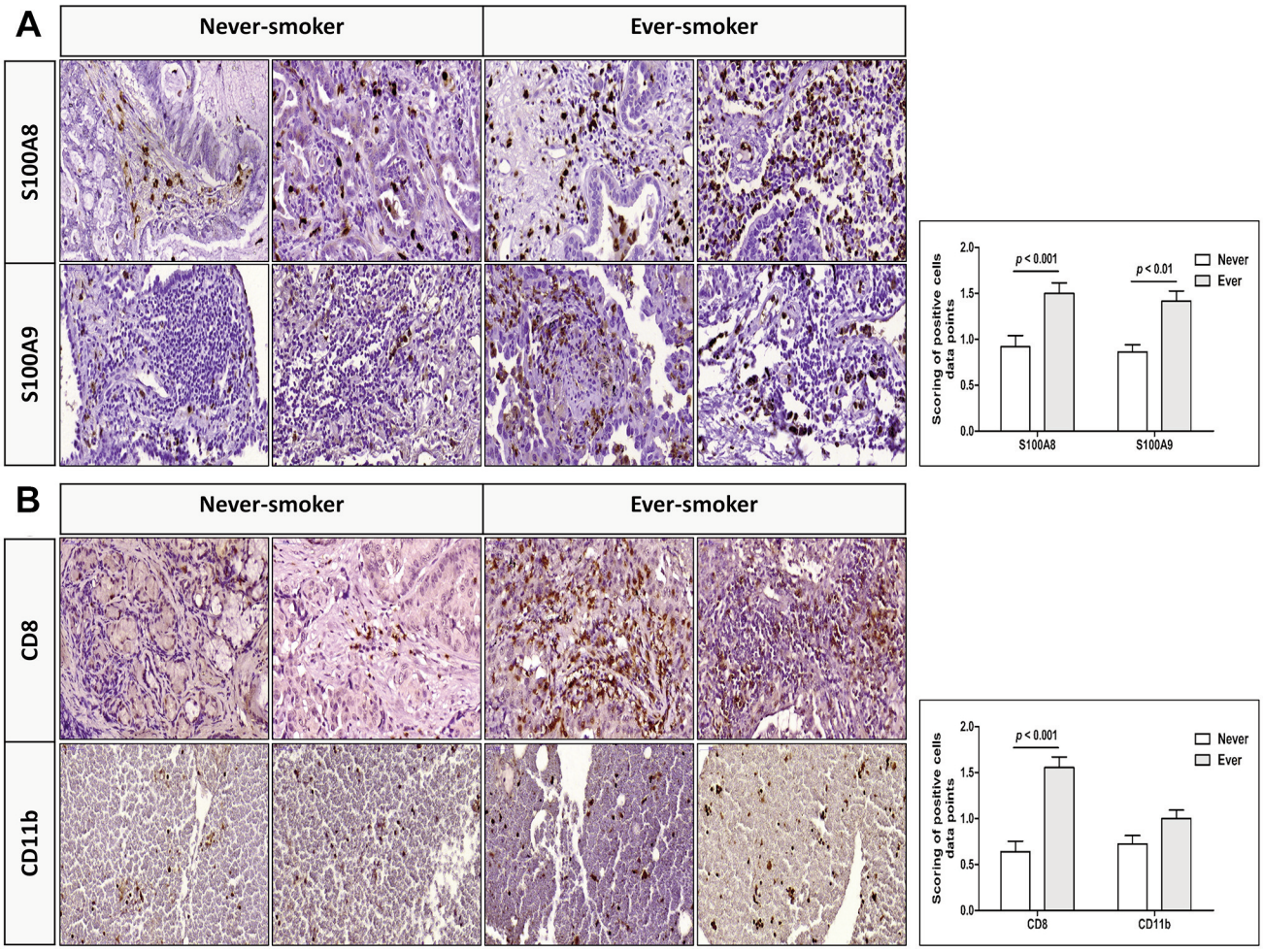

Figure 3. *Immunohistochemical staining patterns of immune and inflammatory cell markers compared to S100A8 and S100A9 in adenocarcinoma. Representative images of IHC staining of (A) S100A8 and S100A9 in the cytoplasm and nucleus and (B) CD8 (anti-CD8 monoclonal antibody) and CD11b (anti-CD11c monoclonal antibody) as inflammatory cell markers. The scoring scale for the semiquantitative analysis of positive cell proportion is presented. Significant differences are indicated as **p<0.01 and ***p<0.001 compared to the never-smoked group (original magnification, ×400).*

help us gain a better understanding of the complexities of cancer metastasis and other inflammatory diseases at the molecular level (30). Anti-inflammatory compounds, such as aspirin, celecoxib, and inhaled corticosteroids, have been shown chemo-preventive effects in tobacco-induced carcinogenesis (31). Based on the present results, blocking the S100A8/A9-RAGE pathway might be investigated as a chemo-preventive agent for populations with smoking-related lung disease, especially COPD. Tasquinimod, a novel oral quinoline-3-carboxamide derivative, had multiple effects on the tumor microenvironment as an anti-cancer agent (32), targeting MDSCs and modulating local tumor immunity by blocking the interaction between S100A8/A9 and its receptors.

Conclusion

In conclusion, the S100A8/9-RAGE pathway and CD8 expression were increased in smoking-related NSCLC patients. The S100A8/9-RAGE pathway could be a promising biomarker for chronic airway inflammation and carcinogenesis in smoking-related lung diseases. The biological functions of S100A8/A9 in the tumor immune microenvironment might be further explored for therapeutic challenges.

Funding

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (NRF-2020R1F1A1066281 and 2021R1A2C2009932).

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

CDY and SHL conceived and planned the experiments. SBC and IKK carried out the experiments and planned and carried out the simulations. CDY and SHL contributed to sample preparation. IKK and SHL contributed to the interpretation of the results. SBC and CDY wrote the manuscript. All Authors provided critical feedback and helped to shape the research, analysis, and manuscript.

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Received August 4, 2023
Revised August 21, 2023
Accepted August 28, 2023