

# Functional analysis of CD14<sup>+</sup>HLA-DR<sup>-low</sup> myeloid-derived suppressor cells in patients with lung squamous cell carcinoma

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**Abstract.** Immunomodulatory therapy is a potential effective treatment for advanced cancer that may provide an alternative to chemotherapy, which patients can experience adverse side effects to. Myeloid-derived suppressor cells (MDSCs) have been demonstrated to cause T-cell tolerance in rodents and humans; however, little is known about the role of MDSCs in squamous cell carcinoma. In the present study, the role of MDSCs in lung squamous cell carcinoma was investigated. Peripheral blood from 78 patients with lung squamous cell carcinoma and 30 healthy controls was examined for the presence and function of human MDSCs, denoted as monocyte differentiation antigen CD14-positive HLA class II histocompatibility antigen DR-negative/low (CD14<sup>+</sup>HLA-DR<sup>-low</sup>) cells by flow cytometry. The sorted T-cell surface glycoprotein CD3 (CD3)<sup>+</sup> cells and CD14<sup>+</sup>HLA-DR<sup>-low</sup> cells were subsequently co-cultured with a tumor cell line (NCI-H226). T-cell apoptosis was detected using annexin-V-fluorescein isothiocyanate and 7-aminoactinomycin D. Interferon- $\gamma$  (IFN- $\gamma$ ) levels were detected using an ELISA. The frequency of MDSCs in the peripheral blood mononuclear cells (PBMCs) from patients with lung squamous cell carcinoma was significantly higher compared with that of the healthy controls ( $P<0.05$ ), whereas the frequency of T-cell surface glycoprotein CD4 (CD4)<sup>+</sup> T cells and CD8<sup>+</sup> T cells in PBMCs was significantly decreased ( $P<0.05$ ). In an MDSC/CD8<sup>+</sup> co-culture system, the proportion of CD8<sup>+</sup> T-cell apoptosis significantly increased with the increase in ratio of MDSCs ( $P<0.05$ ), while the proportion of tumor cell apoptosis significantly decreased ( $P<0.05$ ). The concentration of IFN- $\gamma$  significantly decreased with the increase in MDSCs ( $P<0.05$ ). Therefore, MDSCs participate

in the immune escape of lung squamous cell carcinoma, and may provide a possible therapeutic strategy for the treatment of this disease.

## Introduction

Lung cancer is the most common cancer worldwide, with estimates revealing that almost half of all new lung cancer cases occur in Asia, the majority of them in China. Due to the high prevalence of smoking in China, the rate of lung cancer is higher than that of the majority of European and American countries (1). In addition, due to the high prevalence of smoking, ~30% of lung cancer diagnoses are classified as the squamous histopathological subtype (2). In total, ~80% of patients with lung cancer in China exhibit metastases either at the time of presentation or later in the course of the disease, leading to a high mortality rate (3).

Myeloid-derived suppressor cells (MDSCs), a type of immunosuppressive cell, have previously been demonstrated to serve a role in carcinoma (4). Human MDSCs are a heterogeneous population composed of cells at several differentiation stages of the myeloid lineage (5). Different types of tumors harbor distinct subsets of MDSCs, which can be further divided into granulocytic cluster of differentiation antigen 15-positive HLA class II histocompatibility antigen DR-negative/low (CD15<sup>+</sup>HLA-DR<sup>-low</sup>) and monocytic CD14<sup>+</sup>HLA-DR<sup>-low</sup> monocytic MDSC subsets (6). A recent study identified the existence of a monocytic subset of MDSCs with the CD14<sup>+</sup>HLA-DR<sup>-low</sup> phenotype that suppresses the proliferation of T cells (7).

The purpose of the present study was to investigate the proportion of peripheral CD14<sup>+</sup>HLA-DR<sup>-low</sup> MDSCs in patients with different stages of lung squamous cell carcinoma, and to investigate the association between different tumor stages and MDSC function.

## Materials and methods

**Patients and healthy donors.** A total of 78 patients (67 male and 11 female) diagnosed from January 2014 to October 2015 with lung squamous cell carcinoma at NanFang Hospital of Southern Medical University (Guangzhou, China) were enrolled. The patients were aged between 48 and 72 years old

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(mean, 58.4 years old). The diagnosis and stage classification of these patients were performed according to the American College of Chest Physicians guidelines released in 2013 (8,9). None of the patients had received chemotherapy or surgery prior to the blood sample being taken. Patients with autoimmune diseases, infectious diseases, multi-primary cancers and other serious diseases were excluded from the current study. All patients were divided into four stages according to the tumor-node-metastasis (TNM) diagnostic criteria (10). Among them, there were 0 patients with stage I, 15 patients with stage II, 37 patients with stage III and 26 patients with stage IV lung squamous cell carcinoma. As the healthy control, 30 healthy volunteers were enrolled in the current study. Blood samples were collected from the aforementioned patients and healthy controls. The current study was approved by the Ethics Committee of NanFang Hospital of Southern Medical University (Guangzhou, China). Written informed consent was obtained from each patient and healthy donor.

**Cell isolation and sorting.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples using Ficoll-Hypaque density gradient centrifugation at  $2,500 \times g$  for 20 min at 22°C. MDSCs were isolated from the PBMCs using Miltenyi Macs kit for CD14<sup>+</sup> and HLA-DR<sup>-</sup> (cat. no. 130-091-632; Miltenyi Biotech, Inc., Cambridge, MA, USA), according to the manufacturer's protocol, followed by analysis using a BD FACS Aria™ cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). The purity of the MDSCs was >90%, which was derived using flow cytometry software FlowJo 7.6.1 (FlowJo LLC, Ashland, OR, USA). The CD3<sup>+</sup> T cells were separated from the PBMCs via CD3<sup>+</sup> selection using a MidiMACS™ separator unit (Miltenyi Biotech, Inc.), according to the manufacturer's protocol. The purity of the CD3<sup>+</sup> T cells was >95%.

**Flow cytometry to determine the frequency of CD14<sup>+</sup>HLA-DR<sup>-low</sup> cells in PBMCs from patients.** Multicolor fluorescence-activated cell sorting (FACS) analysis was performed using the following antibodies: Anti-CD14 (560634, 20 µg/ml), anti-HLA-DR-PerCp (552764, 10 µg/ml), anti-CD3-APC (565119, 5 µg/ml), anti-CD4-PE (562281, 20 µg/ml) and anti-CD8-FITC (555366, 20 µg/ml), all supplied by BD Pharmingen, San Diego, CA, USA. Flow cytometry was performed using a FACS Calibur™ flow cytometer (BD Biosciences), according to a previously described method (11). Analysis of the FACS data was performed using FlowJo software (version X.0.7; TreeStar, Inc., Ashland, OR, USA). Isotype-matched antibodies were used with all the samples as controls.

**Apoptosis assay.** The CD4<sup>+</sup> and CD8<sup>+</sup> T cells were co-cultured with MDSCs in the upper compartment of a Transwell plate (EMD Millipore, Billerica, MA, USA) at different ratios (10,000:0, 10,000:1,000, 10,000:5,000, 10,000:10,000 cells) and treated with monoclonal antibodies anti-CD3 (catalog no. 555337; BD Biosciences; 10 µg/ml) for 48 h at 37.0°C and anti-CD8 (catalog no. 557084; BD Biosciences; 20 µg/ml) for 48 h at 37°C. The same proportions of NCI-H226 tumor cells (10,000 cells/well) were cultured in the lower compartment of the Transwell plate at 37°C (Shanghai Shun Biotechnology,

Table I. Clinicopathological characteristics of patients with lung squamous cell carcinoma and healthy controls.

Clinicopathological characteristics	Healthy controls	Patients with lung squamous cell carcinoma
Total, n	30	78
Age, years (mean ± SD)	58.4±8.9	63.4±9.2
Gender (male/female)	26/4	68/10
Tumor-node-metastasis stage		
II	ND	15
III	ND	37
IV	ND	26
SCC antigen, µg/l (mean ± SD)	ND	23.8±1.5
CEA, ng/l (mean ± SD)	ND	135.9±34.1

SCC, squamous cell carcinoma-associated; CEA, carcinoembryonic antigen; SD, standard deviation; ND, no data.

Shanghai, China). Following incubation for 48 h, the cells were collected and stained with annexin-V-fluorescein isothiocyanate and 7-amino-actinomycin D (eBioscience, Inc., San Diego, CA, USA), respectively. CD3<sup>+</sup> cells were stained with anti-CD8-PerCp (catalog no. 560662; 0.5 mg/ml; BD Biosciences) for 20 min at 20°C to analyze the apoptosis of CD8<sup>+</sup> cells. IFN-γ in the supernatant was tested using an ELISA kit (RapidBio Laboratory, Calabasas, CA, USA), according to the manufacturer's protocol.

**Statistical analysis.** All statistical analyses were performed using SPSS software (version 6; SPSS, Inc., Chicago, IL, USA). Comparisons between different groups were analyzed using a Mann-Whitney U test. All data are presented as the mean ± standard deviation.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Patient clinicopathological characteristics.** The clinicopathological characteristics and clinical stage of the 78 patients and 30 healthy controls are illustrated in Table I. There was no significant difference between the age and gender of the study subjects in the control group and the lung squamous cell carcinoma group. All patients were diagnosed with lung squamous cell carcinoma by clinical methods, imaging, bronchoscopy and pathology. The mean levels of squamous cell carcinoma (SCC) antigen and carcinoembryonic antigen (CEA) in the patients with lung squamous cell carcinoma were  $23.8 \pm 1.5$  µg/l and  $135.9 \pm 34.1$  ng/l, respectively. The normal reference range of SCC is  $\leq 1.5$  µg/l and the normal reference range of CEA is  $\leq 5$  ng/ml.

**Frequency of MDSCs is significantly increased in patients with lung squamous cell carcinoma compared with that of healthy controls.** MDSC frequency in the peripheral blood was analyzed using flow cytometry following density gradient centrifugation.

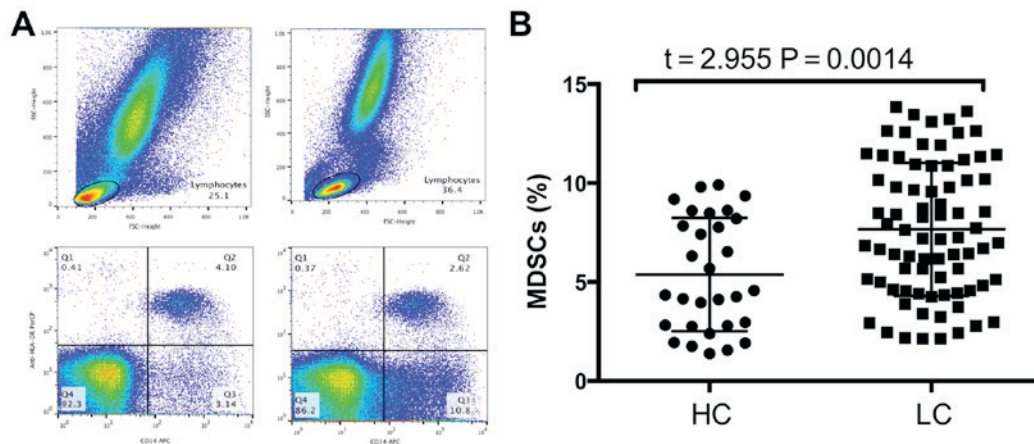


Figure 1. Flow cytometry analysis to quantify the frequency of MDSCs in patients with lung squamous cell carcinoma. (A) Gating strategy used for the identification of CD14<sup>+</sup> HLA-DR<sup>-low</sup> MDSCs and representative flow cytometry data. (B) Quantification of the frequency of MDSCs from 78 patients with lung squamous cell carcinoma and 30 healthy controls. MDSCs, myeloid-derived suppressor cells; CD14, monocyte differentiation antigen CD14; HLA-DR, HLA class II histocompatibility antigen DR; HC, healthy control; LC, lung squamous cell carcinoma.

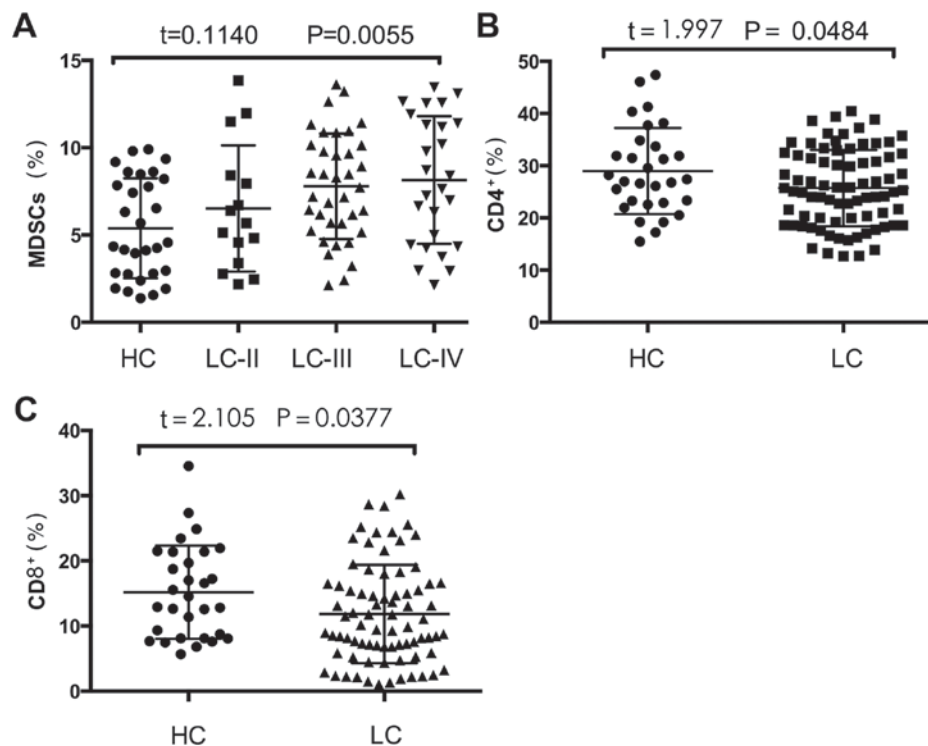


Figure 2. Frequency of MDSCs positively correlates with TNM stage in patients with lung squamous cell carcinoma. (A) Frequency of MDSCs in the peripheral blood of patients with different TNM stages (II, III and IV) of lung squamous cell carcinoma. (B) Frequency of CD4<sup>+</sup> cells in healthy controls and lung squamous carcinoma patients. (C) Frequency of CD8<sup>+</sup> cells in healthy controls and lung squamous carcinoma patients. MDSCs, myeloid-derived suppressor cells; HC, healthy control; LC, lung squamous cell carcinoma; CD4, T-cell surface glycoprotein CD4; CD8, T-cell surface glycoprotein CD8; TNM, tumor-node-metastasis.

To exclude debris and dead cells, the lymphocytes were selected. Next, CD14<sup>+</sup> cells were selected, followed by gating of the HLA-DR<sup>-low</sup> population (Fig. 1A). The frequency of MDSCs in the peripheral blood of patients with lung squamous cell carcinoma was significantly higher compared with that of healthy controls ( $5.38 \pm 0.52$  vs.  $7.664 \pm 0.38\%$ ;  $P = 0.0014$ ; Fig. 1B).

*Frequency of MDSCs positively correlates with disease stage in patients with lung squamous cell carcinoma.* In order to further reveal the role of MDSCs, the TNM staging

method, which is based on tumor size, lymph node metastasis, tumor-localized metastasis and tumor-distant metastasis, was used to stage each patient. The frequency of MDSCs was positively correlated with TNM stage (Fig. 2A). The frequencies of MDSCs in patients with stage II, III and IV lung squamous cell carcinoma were  $6.51 \pm 3.61$ ,  $6.51 \pm 2.97$  and  $6.82 \pm 3.45\%$ , respectively ( $P = 0.0055$ ; Fig. 2A).

*Frequencies of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in PBMCs from patients with lung squamous cell carcinoma are significantly*

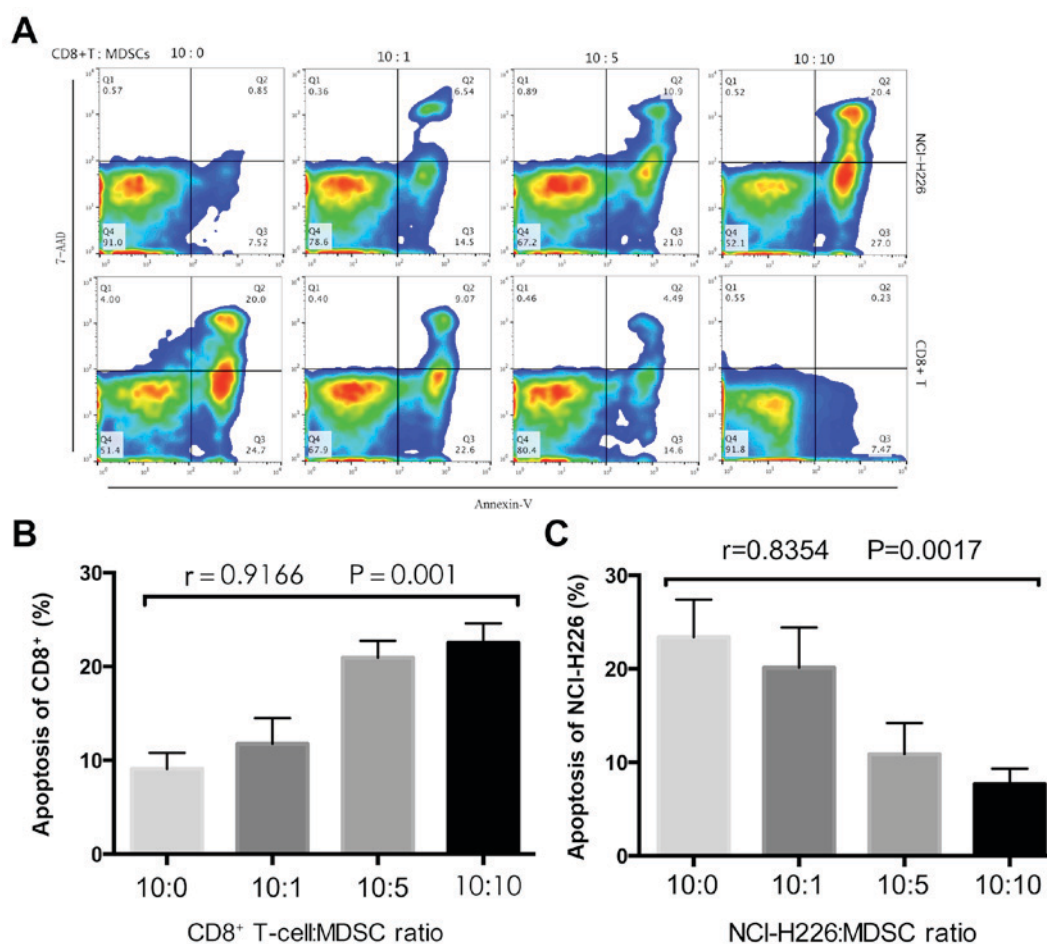


Figure 3. MDSCs inhibit T cell cytokine secretion *in vitro*. (A) Analysis of NCI-H226 and CD8<sup>+</sup> cell apoptosis. (B) Statistical analysis of apoptosis of CD8<sup>+</sup> T cells and NCI-H226 cells. (C) Statistical analysis of apoptosis of NCI-H226 cells.

decreased compared with those of healthy controls. To further investigate the different functions of the MDSCs in patients and healthy controls, the percentages of circulating CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells were measured (Fig. 2B and C). The percentages of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in the PBMCs of patients with lung squamous cell carcinoma were significantly decreased compared with those of healthy controls ( $28.97 \pm 1.51$  vs.  $25.71 \pm 0.83\%$ ,  $P = 0.0484$ ; and  $15.20 \pm 1.31$  vs.  $11.84 \pm 0.85\%$ ,  $P = 0.0377$ , respectively; Fig. 2B and C).

**MDSCs inhibit T-cell cytokine secretion *in vitro*.** To investigate the inhibitory effects that MDSCs exhibit on CD8<sup>+</sup> T cells, MDSCs were sorted and co-cultured with CD8<sup>+</sup> T cells and tumor cells at the indicated ratios (Fig. 3). Following 48 h, the CD8<sup>+</sup> T cells and tumor cells were labeled with Annexin-V-FITC and 7-AAD respectively, followed by detection using flow cytometry. An ELISA was performed to measure IFN- $\gamma$  levels in the co-culture supernatant. The proportion of CD8<sup>+</sup> T-cell apoptosis was significantly increased as the proportion of MDSCs increased ( $P = 0.001$ ; Fig. 3B), whereas the proportion of tumor cell apoptosis significantly decreased ( $P = 0.0017$ ; Fig. 3C). The concentration of IFN- $\gamma$  significantly decreased with the increase in MDSCs ( $P = 0.0016$ ; Fig. 4), which implies that the MDSCs inhibit T cell cytokine secretion.

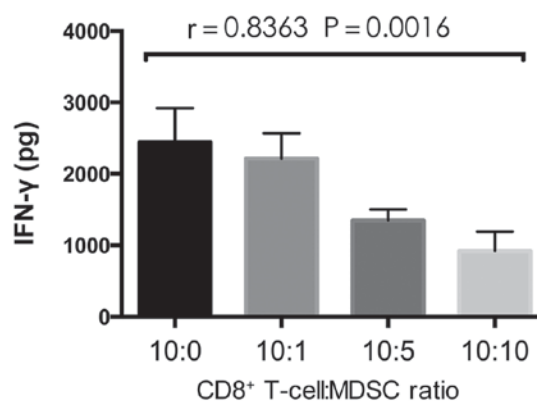


Figure 4. Concentration of IFN- $\gamma$  in the supernatant of the T-cell surface glycoprotein CD8<sup>+</sup> T cell/MDSC co-culture at the indicated ratios. MDSCs, myeloid-derived suppressor cells; IFN- $\gamma$ , interferon- $\gamma$ .

## Discussion

The human immune system has evolved over millions of years and can protect the body from pathogens, including bacteria, and parasites (12). Designing an immunotherapy that can enhance the anticancer effects of the immune system remains a



challenge and immunotherapies have had little success in clinical trials (13). It has previously been demonstrated that tumor size is not significantly affected following the administration of immunotherapy, which may be due to certain cell types that suppress the immune response (14,15). An optimistic trend in the treatment of lung disease, which may change the immune suppressive effect, is emerging (16). Immunotherapies are designed to stimulate the immune system in order to restore its anticancer effects (17,18). The purpose of the current study was to evaluate whether lung squamous cell carcinoma cells are affected by MDSCs, as there is currently little information on changes in MDSC proportion in patients with tumors. A number of recent studies, performed independently in patients with non-small cell lung cancer (NSCLC) and other carcinomas, demonstrated that these cells are immunosuppressive and that their frequency is upregulated in carcinoma (19-21).

Previous studies have demonstrated the functions of human MDSCs in hepatocellular, renal carcinoma and prostate cancer types, among others (22,23). The proliferation and aggregation of MDSCs in human malignant neoplasms may have an effect on tumor progression and prognosis. The definition of these immunosuppressive cells in patients is problematical, since there is no human homolog of Gr-1 marker, and no correlation between phenotype and immune suppressive properties has been reported (11,24). Identifying MDSCs may aid in developing anticancer treatments that target these cell populations. MDSCs are a heterogeneous group of cells that include monocytic (M)-MDSCs, polymorphonuclear MDSCs and immature myeloid cells. These three subsets can express different combinations of myeloid markers that are associated with different differentiations of myeloid cells (CD14, CD15, HLA-DR, CD33, CD11b, CD15 and CD16), and can possess the immunosuppressive activity of MDSCs (25).

M-MDSCs were the first subtype of human MDSCs to be identified in the peripheral blood of melanoma patients, and are defined as CD14<sup>+</sup> and HLA-DR<sup>-/low</sup> cells (26). M-MDSCs have also been identified in a number of other cancer types, including renal cell carcinoma, hepatocellular carcinoma and advanced NSCLC (27). In the present study, following the exclusion of debris and granulocytes, CD14<sup>+</sup> and HLA-DR<sup>-/low</sup> cells were selected, as these cells have been widely studied. The frequency of these cells has been reported to be elevated in a number of cancer types (28); however, further studies are required in patients with lung squamous cell carcinoma. Data from the present study demonstrated that the frequency of MDSCs in the PBMCs of 78 patients with lung squamous cell carcinoma was significantly increased compared with that of healthy controls. Additionally, the frequency of MDSCs was associated with TNM stage, and the levels of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were significantly decreased in patients with lung squamous cell carcinoma compared with healthy controls. Previous studies have demonstrated that the decreased number of lymphocytes described in patients with cancer is partially due to the immunosuppressive effects of MDSCs (29,30).

According to previous studies, MDSCs mediate immunosuppression through a number of molecular mechanisms. MDSCs deplete essential metabolites for T lymphocytes through the activation of arginase-1 and nitric oxide synthase 2 (31). High levels of reactive oxygen species affect T cells by down-regulating T-cell surface glycoprotein CD3  $\zeta$  chain expression

and reducing cytokine secretion. MDSCs interfere with T cell migration and viability by expressing the metalloproteinase disintegrin and metalloproteinase domain-containing protein 17 that is able to cleave the integrin CD62L on T cells. MDSCs promote the clonal expansion of antigen-specific natural regulatory T cells (Tregs) and induce the conversion of CD4<sup>+</sup> T cells into induced Tregs through the release of transforming growth factor- $\beta$  (32). In order to further verify the immunosuppressive effect of MDSCs in lung squamous carcinoma cell immunity, MDSCs from patients with lung squamous cell carcinoma and healthy controls were sorted and subsequently cultured with NCI-H226 cells. As the ratio of MDSCs increased, the proportion of CD8<sup>+</sup> T cell apoptosis significantly increased, whereas NCI-H226 cell apoptosis significantly decreased. Additionally, the concentration of IFN- $\gamma$  significantly decreased with the increase in MDSCs, which implies that MDSCs inhibit T cell cytokine secretion. This confirms the immunosuppressive effect of MDSCs in lung squamous cell carcinoma (33). These results may aid in developing novel treatments that inhibit malignant neoplasm progression and metastasis. It has previously been reported that it is possible to use MDSCs as a therapeutic target (34).

The present study investigated the proportion of peripheral CD14<sup>+</sup>HLA-DR<sup>-/low</sup> MDSCs in patients with different stages of lung squamous cell carcinoma, and the association between different tumor stages and MDSC function. The frequency of MDSCs is significantly increased in patients with lung squamous cell carcinoma. The frequencies of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells in PBMCs from patients with lung squamous cell carcinoma were significantly decreased compared with those from the healthy controls. MDSCs inhibit T-cell cytokine secretion *in vitro*. In conclusion, MDSCs participate in the immune escape of lung squamous cell carcinoma, and may provide a possible therapeutic strategy for the treatment of this disease.

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