Abstract. Lung cancer causes severe health problems worldwide and, in China, besides being the principal cause of mortality among urbanites, it is the second leading cause of mortality in the rural population, preceded by hepatocellular carcinoma. Between 80 and 85% of lung cancer is classified as non-small cell lung cancer (NSCLC). The high mobility group box 1 (HMGB1) protein serves an important function in the tumor microenvironment and antitumor response, and may be targeted by microRNA (miR). In the present study, miR-129-5p was identified to target HMGB1 and miR-129-5p exhibited low expression levels in NSCLC tissues. Overexpression of miR-129-5p inhibited cell proliferation and promoted cell apoptosis. In conclusion, the results of the present study suggested the inhibitory function of miR-129-5p and revealed a novel therapeutic target for further investigation.

Introduction

Lung cancer is the leading cause of cancer-associated mortality in China (1). Two major types of lung cancer exist: Non-small cell lung cancer (NSCLC), which accounts for between 80 and 85% of lung cancer cases, and small cell lung cancer (SCLC), which accounts for between 10 and 15% of lung cancer cases (2). NSCLCs include adenocarcinoma, large cell carcinoma, bronchioloalveolar carcinoma and squamous cell carcinoma (3,4).

It has been reported that the high mobility group box 1 (HMGB1) protein is involved in the immune response of tumors (5). The HMGB1 protein was first identified as a highly conserved nuclear protein that serves a pivotal function in chromatin organization and transcriptional regulation (6,7). In addition, HMGB1 also serves important functions in the immune response (8). Preclinical data revealed that dying tumor cells emit certain danger molecular signals, known as damage-associated molecular patterns (DAMPs) (9-14). In brief, DAMPs are intracellular sequestered biomolecules that evade recognition by the immune system under normal physiological conditions (15). However, under conditions of cellular stress or injury, these molecules are actively secreted by stressed immune cells or released from dying cells, and trigger a non-infectious inflammatory response (16). HMGB1 is a typical DAMP biomolecule, as HMGB1 is released from dying cells and can trigger an immune response (17). A previous study demonstrated that, during chemotherapy or radiotherapy, HMGB1 was released from dying cells and stimulated dendritic cells (DCs) via Toll-like receptor 4 (TLR4) (5). DCs require signaling through TLR4 and its adaptor myeloid differentiation primary response 88 (MyD88) for efficient processing and cross-presentation of antigens from dying tumor cells (5).

MicroRNAs (miRNAs/miRs) serve multiple functions, including promoting cellular proliferation and apoptosis, in the pathogenesis of lung cancer (18-24). HMGB1 may be a target for miRNAs. A previous study demonstrated that miR-218 is able to suppress cell proliferation and invasion, and promotes the apoptosis of pancreatic cancer cells by targeting HMGB1 (25).

In the present study, the function of miR-129-5p in lung cancer was examined and it was demonstrated that miR-129-5p targets HMGB1. The results of the present study may provide novel insights into the molecular mechanism for lung cancer progression.

Patients and methods

Tissue samples. A total of 10 NSCLC tissue samples were collected from The Pulmonary Department at Sichuan Cancer Center (Chengdu, China). Of the ten patients included in the study, 4 were male and 6 were female, with a mean age of 60 years (range, 43-77 years). The 10 NSCLC tissue samples were collected between January 2011 and May 2012. The exclusion criteria was any patient who had been diagnosed with another type of cancer. The protocol for the collection and use of human tissues in the present study was evaluated and approved by the Ethics Committee of Sichuan Cancer Hospital (Chengdu, China). All patients enrolled in the present study provided written informed consent, and all specimens were handled and blinded as required by the legal standards of China. All NSCLC tissue samples were
evaluated and confirmed by a senior pathologist at Sichuan Cancer Center. The clinical information are presented in Table I.

**Cell culture.** The NSCLC cell lines A549 and SPC-A-1 were obtained from the Cell Bank of Sichuan University (Sichuan, China). All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified incubator with 5% CO$_2$.

**Detection of miR-129-5p in tissues and cells.** The levels of miR-129-5p in tissues and cells were determined using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Briefly, the total RNA from the tissue samples or cell transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The corresponding cDNA was obtained using the reverse transcription kit (cat. no. 12574018, Thermo Fisher Scientific, Inc.). The expression levels were calculated with the $2^{-\Delta\Delta Cq}$ method (26).

**Overexpression and downregulation of miR-129-5p in NSCLC cells.** The miR-129-5p levels in the A549 and SPC-A-1 cells were increased and decreased by miR-129-5p mimic and miR-129-5p antisense oligonucleotide (ASO), respectively. The miR-129-5p mimic and were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) (27-30). The following primer sequences were used: miR-129-5p forward, 5′-GGG GGC TTG GCAGCACA-3′ and reverse, 5′-AACGCTTCAAGATT TGCGT-3′.

**Cell proliferation assay.** Cell proliferation was assessed using the MTT assay. Briefly, A549 and SPC-A-1 cells were seeded at 1x10^5 cells/well and serum-starved for 6 h pre-transfection. The 3′-untranslated region (3′-UTR) of HMGB1 and mutated controls were cloned and inserted into a reporter plasmid (500 ng) and the pGL3-control (100 ng) (Promega Corporation, Madison, WI, USA). miR-129-5p mimics were then transfected into A549 cells containing wild-type or mutant 3′-UTR plasmids with Lipofectamine 2000. Cells were harvested and luciferase activity was analyzed after 24 h using the Dual-Luciferase Reporter assay system (Promega Corporation). Luciferase activity was normalized to Renilla luciferase activity. Mutants of HMGB1 3′-UTR were generated using a Site-Directed Mutagenesis kit (Promega Corporation).

**Cell apoptosis analysis.** A549 or SPC-A-1 cell suspensions (5x10^4 cells/ml) and Annexin V-FITC (Abcam, Shanghai, China) binding buffer was prepared. An Annexin V-FITC Apoptosis Detection kit (cat. no. APOAF-20TST; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used according the manufacturer’s protocol. Annexin V-fluorescein isothiocyanate (FITC; 0.5 mg/ml) was added to the cell mixture and incubated at room temperature for 15 min. Subsequently, propidium iodide (PI; 0.1 µg/ml; Abcam) was added for 5 min at room temperature, and samples were analyzed on a fluorescence-activated cell sorting analyzer instrument using the 488 nm excitation wavelength (argon-ion laser or solid state laser) and emission detected was at 530 nm (green, FITC) and 575-610 nm (orange, PI) (28). Data were analyzed with BD FACSuite™ software (23-12943; BD Biosciences, San Jose, CA, USA).

**Prediction of the possible targets of miR-129-5p.** TargetScan software (www.targetscan.org) was used to predict the possible targets of miR-129-5p.

**Dual-luciferase reporter assays.** A549 cells were seeded at 1x10^4 cells/well and serum-starved for 6 h pre-transfection. The 3′-untranslated region (3′-UTR) of HMGB1 and mutated controls were cloned and inserted into a reporter plasmid (500 ng) and the pGL3-control (100 ng) (Promega Corporation, Madison, WI, USA). miR-129-5p mimics were then transfected into A549 cells containing wild-type or mutant 3′-UTR plasmids with Lipofectamine 2000. Cells were harvested and luciferase activity was analyzed after 24 h using the Dual-Luciferase Reporter assay system (Promega Corporation). Luciferase activity was normalized to Renilla luciferase activity.

**Western blotting.** A549 cells were frozen and lysed in lysis buffer (150 mM NaCl, 50 mM Tris/HCl, 1% Triton X-100 and 0.1% SDS) with protease inhibitors cocktail (cat. no. ab65621; Abcam). Protein concentration was determined using a BCA protein assay (cat. no. ab146331; Abcam). Subsequently, 20 µg total protein from the cell lysate was loaded onto

### Table I. Characteristics of the 10 patients with non-small cell lung cancer.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age, years</th>
<th>Sex</th>
<th>TNM stage</th>
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<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>Female</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
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<td>10</td>
<td>73</td>
<td>Female</td>
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a 10% SDS-PAGE gel. The protein was then transferred to PVDF membrane and the membrane was blocked with 5% milk for 1 h at room temperature. For the HMGB1 western blot analysis, anti-HMGB1 antibodies (cat. no. ab79823; Abcam) were used at a dilution of 1:1,000 at 4°C overnight. β-actin was used as an internal reference (cat. no. ab179467; Abcam). The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2,000; cat. no. ab150077; Abcam) at room temperature for 2 h. Proteins were detected by Enhanced Chemiluminescence Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). Images were analyzed using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All experiments were performed three times. Results are presented as the mean ± standard deviation. A two-tailed Student's t-test was used to analyze the mean value between two groups; One-way analysis of variance was used to test the mean value among three or more groups with post hoc contrasts by Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference. All calculations were performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA).

Results

miR-129-5p is expressed at low levels in lung cancer tissues. First, the miR-129-5p expression levels in 10 NSCLC tissue samples and their corresponding normal adjacent tissue samples were analyzed using RT-qPCR. Tumor tissue samples exhibited lower miR-129-5p levels compared with their corresponding normal adjacent tissues (Fig. 1A). The mean expression levels of miR-129-5p in all 10 tumor tissue samples and normal tissue samples were determined, and were revealed to be significantly lower in tumor tissue samples compared with normal tissue samples (Fig. 1B). These results suggest that miR-129-5p may exhibit a tumor suppressor function in NSCLC.

Overexpression of miR-129-5p inhibits cell proliferation and promotes cell apoptosis. To investigate the function of miR-129-5p in vitro, the levels of miR-129-5p in A549 and SPC-A-1 cells were analyzed using RT-qPCR, normal lung tissues were used as control. A549 and SPC-A-1 cells expressed significantly lower levels of miR-129-5p compared with normal tissues (Fig. 2A). Additionally, miR-129-5p mimic was transfected into A549 and SPC-A-1 cells, and it was identified that the miR-129-5p mimic effectively upregulated the miR-129-5p levels in A549 and SPC-A-1 cells (Fig. 2B). As the miR-129-5p mimic was able to increase the miR-129-5p level in vitro, cell proliferation following transfection with the miR-129-5p mimic was determined, and it was identified that the upregulation of miR-129-5p inhibited cell proliferation in A549 and SPC-A-1 cells (Fig. 2C). The effect of the miR-129-5p mimic on cell apoptosis was investigated and transfection of the miR-129-5p mimic was identified to increase the rate of apoptosis (Fig. 2D).

Downregulation of miR-129-5p promotes cell proliferation. miR-129-5p ASO was transfected into A549 and SPC-A-1 cells to decrease the miR-129-5p levels. The miR-129-5p levels in A549 and SPC-A-1 cells 24 h after miR-129-5p ASO transfection revealed that miR-129-5p were significantly inhibited by miR-129-5p ASO (Fig. 3A). Additionally, cell proliferation was determined using the MTT assay and it was identified that miR-129-5p ASO transfection significantly increased cell proliferation (Fig. 3B).

miR-129-5p inhibits HMGB1 expression in lung cancer cells. HMGB1 is an important immune factor and one of the genes targeted by miR-129-5p (1,6-8). The present study identified the binding site of miR-129-5p on the HMGB1 gene using TargetScan software (Fig. 4A). To confirm whether miR-129-5p targeted HMGB1, a mutant of the 3'-UTR of HMGB1 was generated and the mutant and wild-type versions were cloned into luciferase reporter plasmids. The miR-129-5p mimic and mutant version were co-transfected into A549 cells. At 24 h
Later, it was identified that the miR-129-5p mimic decreased the luciferase values of the 3'UTR of HMGB1 (wild-type version), but not that of the mutated version of the 3'UTR of HMGB1 (Fig. 4B). Furthermore, the miR-129-5p mimic was transfected into A549 cells and, 48 h later, the HMGB1 protein levels were evaluated using western blot analysis. The results identified that the miR-129-5p mimic inhibited HMGB1 protein expression (Fig. 4C).

Discussion

The present study investigated the function of miR-129-5p in NSCLC and identified that miR-129-5p exhibited tumor suppressor activity against NSCLC via HMGB1. The function of miR-129-5p has previously been investigated in various types of cancer. In gastric cancer, miR-129-5p was identified to be downregulated and involved in the migration and invasion of gastric cancer cells by targeting interleukin-8 (31). In laryngeal cancer, the upregulation of miR-129-5p inhibited laryngeal cancer cell proliferation, invasiveness and migration by affecting signal transducer and activator of transcription 3 expression (32), and in breast cancer, miR-129-5p attenuates irradiation-induced autophagy and decreases radioresistance of breast cancer cells by targeting HMGB1 (33). The results also indicated that miR-129-5p serves a function as an inhibitor of tumor growth.

HMGB1 may activate and induce the maturation of DCs (34). Accordingly, HMGB1 is one of the links between tumor cells and the immune system of the host (35). Recent studies have identified that, in NSCLC, DCs are in contact with tumor cells (36), and tumor-infiltrating lymphocytes (TILs) are observed in the peritumoral zones (37). HMGB1 produced by tumor cells recruits DCs, which associate with an increased number of TILs (38). Furthermore, HMGB1 may be released from dying cells and stimulate DCs (5). DCs required signaling through TLR4 and its adaptor MyD88 for the efficient processing and cross-presentation of antigens from dying tumor cells (5). We hypothesize that HMGB1 may serve an important function in the immune response against tumors and therefore represents a potential immune therapy target in cancer. Accordingly, miR-129-5p may serve a function in the NSCLC immune therapy via HMGB1.

A limitation of the present study was that only ten NSCLC tissue samples were analyzed. The number of patients involved in the study limited the patient survival analysis. Therefore, a larger number of patients should be included in further investigations.

In conclusion, the results of the present study revealed the suppressive function of miR-129-5p in NSCLC and offer a
Figure 3. Suppression of miR-129-5p levels promotes A549 and SPC-A-1 cell proliferation. A549 and SPC-A-1 cells were seeded and then transfected with miR-129-5p ASO. (A) At 24 h after transfection, miR-129-5p expression was determined using the reverse transcription-quantitative polymerase chain reaction. (B) Following miR-129-5p ASO transfection, cell proliferation was determined. Results are presented as the mean ± standard deviation. The experiment was performed at least three times. *P<0.05 vs. NC, or miR-NC. OD, optical density; NC, negative control; ASO, antisense oligonucleotide; miR, microRNA.

Figure 4. HMGB1 is targeted by miR-129-5p in A549 cells. (A) The binding sites and its mutated version of HMGB1 and miR-129-5p. (B) miR-129-5p mimic and plasmid containing WT or mutated 3′-UTR of HMGB1 were transfected into A549 cells. After 48 h, the luciferase activity was measured. (C) The miR-129-5p mimic was transfected into A549 cells, and the abundance of the HMGB1 protein was analyzed by western blotting. The experiment was performed at least three times. *P<0.05 vs. WT. HMGB1, high mobility group box 1; UTR, untranslated region; WT, wild-type; NC, negative control; miR, microRNA.
novel therapeutic target for the treatment of NSCLC that is worthy of further investigation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

GL collected patient data and performed the cell experiments. JX performed reverse transcription-quantitative polymerase chain reaction analysis, western blotting and other molecular experiments. JW contributed to the study design and manuscript writing.

Ethics approval and consent to participate

The protocol for the collection and use of human tissues in the present study was evaluated and approved by the Ethics Committee of Sichuan Cancer Hospital. All patients enrolled in the present study provided written informed consent, and all specimens were handled and blinded as required by the legal standards of China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References