The Study on Newly Developed McAb NJ001 Specific to Non-Small Cell Lung Cancer and Its Biological Characteristics

Shiyang Pan1,2,*, Fang Wang1,2,*, Peijun Huang1,2,*, Ting Xu1,2, Lixia Zhang1,2, Jian Xu1,2, Qing Li1,2, Wenying Xia1,2, Ruihong Sun1,2, Lei Huang1,2, Ying Peng1,2, Xuejun Qin1,2, Yongqian Shu3, Zhibin Hu4, Hongbing Shen4

1 Department of Laboratory Medicine, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, 2 National Key Clinical Department of Laboratory Medicine, Nanjing, China, 3 Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, 4 Department of Epidemiology and Biostatistics, Cancer Center of Nanjing Medical University, Nanjing, China

Abstract

Monoclonal antibody (McAb) is the key tool for cancer immunodiagnostics and immunotherapy. McAb-based immunotherapy that targets tumor antigens has had great achievement. In this study, a cell clone which kept secreting high-titer IgG1-type McAb named NJ001 against human non-small cell lung cancer (NSCLC) cells was obtained. The titer of purified NJ001 was 2 × 10^6. The antigen named SP70 of NSCLC specifically identified by NJ001 was proved to be a protein with the relative molecular mass (Mr) of 70 kDa. The results of immunohistochemical staining indicated that NJ001 could positively react to NSCLC, but weak positively or negatively react to human small-cell lung cancer (SCLC), pulmonary pseudotumor and other epithelial tumors. In soft agar assay, the colony formation efficiency in NJ001 groups decreased in a dose-dependent manner. For the concentration of 100 μg/ml, 200 μg/ml and 400 μg/ml, the inhibition ratio of colony formation was 23.4%, 62.5% and 100% respectively. Meanwhile, NJ001 caused significant reduction in tumor volume and tumor weight compared to control mice in lung cancer xenograft model. The tumor growth inhibition ratio in 200 μg, 400 μg and 800 μg NJ001 groups was 10.44%, 37.29% and 44.04%, respectively. NJ001 also led to cytomorphological changes and induced the apoptosis of human lung adenocarcinoma cell line SPC-A1 significantly. The newly developed NJ001 selectively reacted to NSCLC and exhibited anti-tumor activity both in vitro and in vivo. NJ001 is of great value concerning immunodiagnostics and immunotherapy for NSCLC and holds promise for further research regarding the mechanism underlying tumor progression of NSCLC.

Introduction

Lung cancer is one of the most prevalent cancers and is the leading cause of cancer death due to the lack of a validated or effective screening approach for early detection. This public health burden is evident worldwide with 1.5 million lung cancer related deaths in 2010 [1]. Non-small cell lung cancer (NSCLC) accounts for more than 85% of lung cancer and most patients with NSCLC have advanced disease at diagnosis. The five-year survival rate for breast, colon, and prostate cancer, in which screening tests are available, is four to six times longer than lung cancer. The high morbidity/mortality and failure to achieve an early diagnosis result in the dismal prognosis [2,3].

The therapies for lung cancer are primarily based on traditional modes such as surgical resection, chemotherapy, and radiotherapy; however, the curative effect obtained is less than satisfactory [4–6]. Recently, immunotherapy for cancer has become a method utilized as a follow-up to traditional therapy. Antibodies are becoming a major drug modality due to their high specificity and affinity to targets. Over two dozen therapeutic monoclonal antibodies (McAbs) are currently approved for the treatment of cancer and other human diseases [7–9]. Identification of novel antigens will further improve tumor immunotherapy. Antibody-based immunotherapy that targets tumor antigens or cell surface markers has achieved some success as a cancer therapy, including in NSCLC, with agents such as cetuximab, panitumumab, matuzumab, and trastuzumab [10–14].

In the present study, we produced a monoclonal antibody designated NJ001, generated by immunizing mice with human SPC-A1 lung adenocarcinoma live cell antigen. The anti-tumor activity of McAb NJ001 was exhibited both in vitro and in vivo.
Results

Production and Characterization of NJ001

Upon immunizing BALB/c mice with human lung adenocarcinoma cells, 3 positive monoclonal hybridoma cell lines (NM001, NM004, NM005) were confirmed by repeated indirect cell ELISA testing to continuously produce antibodies and were thus selected for expansion and cloning. Chromosome numbers of each hybridoma karyotype were more than 95. Figure 1A was the karyotype of NM001 hybridoma cell. Purified McAbs from ascites were acquired by Protein A affinity purification. The McAb from NM001 had the highest titre among the three antibodies, reaching 2×10^6. NM001 and NM004 kept secreting IgG1, κ-type McAb, while NM005 secreted IgG2b, κ-type McAb.

Each McAb was characterized by the results of binding to a panel of normal and malignant cells listed in Figure 1B. By ELISA analysis, we determined that McAbs produced by the hybridoma cell lines NM001, NM004 and NM005 exhibited higher binding ratios with the NSCLC cell lines SPC-A1, A549, NCI-520 and NCI-H460, but exhibited generally lower binding ratios in SCLC cell line NCI-H446, other cancer and normal cell lines. NJ001, produced by the hybridoma cell line NM001, was selected for further study because it exhibited the highest specificity for the lung cancer cell lines compared to other tumor cell lines.

Western blot analysis results were consistent (Figure 1C). We could only detect the expression of the protein named SP70 specific to NJ001 in NSCLC cell lines, but not in other cancer cell lines and normal cells.

Indirect immunofluorescence results were shown in Figure 1D. SP70, recognized by NJ001, was localized in the cell membrane and cellular cytoplasm of SPC-A1 cells, whereas the other cell lines exhibited no fluorescence.

Immunohistochemical Analysis

Immunohistochemical analysis results showed that the expression of the SP70 was strong positive in lung adenocarcinoma tissue and squamous lung cancer tissue (Figure 2A, 2B), while weak positive or negative expression was observed in the tissue of SCLC, breast carcinoma, gastric cancer, colon cancer, ovarian cancer and liver cancer (Figure 2C, 2D, 2E, 2F, 2G, 2H). SP70 was not found in the tissues of pulmonary pseudotumor and adjacent non-tumourous lung tissues (Figure 2I, 2J).

SP70 expressions in the tissue of lung adenocarcinoma, squamous lung cancer, SCLC, breast carcinoma, gastric cancer, colon cancer, ovarian cancer, liver cancer, pulmonary pseudotumor and adjacent non-tumorous lung were 58/58, 48/48, 2/21, 3/21, 1/5, 0/5, 0/5, 1/5, 0/25 and 0/8 respectively (Table 1).

Inhibitory Effects of NJ001 on SPC-A1 Proliferation and Colony Formation

The effect of NJ001 on the proliferation of SPC-A1 cells was evaluated via a [3H] thymidine proliferation assay. Compared with the control treated cells, the proliferation level of NJ001 treated SPC-A1 cells significantly decreased after 48 h and 72 h (P<0.001, P<0.001) (Figure 3).

SPC-A1 cells were plated on a soft agar matrix, treated with NJ001 or MCA2849 (irrelevant McAb) 0, 100, 200, 400, 800, or 1000 μg/mL and incubated at the condition of 37°C with 5% CO2. After 14 days, the number of colonies was counted and the representative images were obtained (Figure 4). As shown in Table 2, NJ001 inhibited colony formation in a dose-dependent manner, exhibiting 23.4% inhibition ratio at 100 μg/mL, 62.5% inhibition ratio at 200 μg/mL. When the concentration reached 400 μg/mL or higher, there were no colonies larger than 50 cells, showing a 100% inhibition ratio. However, MCA2849 didn’t inhibit the colony formation of SPC-A1.

These results suggested that NJ001 effectively inhibited SPC-A1 cell proliferation in vitro.

Inhibitory Effects of NJ001 in the Human SPC-A1 Lung Adenocarcinoma Mouse Xenograft Model

In the preliminary study, after 3 weeks of inoculation, the mice were euthanized. The tissues from the injection site in the incubation group and the tumors in the control group were excised. Histopathology showed no tumor growth in tissues of the incubation group and tumor growth in tissues of the control group (Figure 5).

The result of in vivo experiment was shown in Figure 6A. The administration of NJ001 caused varying degrees of reduction in tumor volume compared with the saline-treated control mice. The tumor volumes in the 400 μg and 800 μg NJ001 group were significantly smaller compared to the control group 17 days after inoculation; moreover, the difference persisted to the end of the treatment (P=0.004, P=0.003). After 3 weeks of treatment, tumors were excised and weighed. In 200 μg, 400 μg and 800 μg NJ001 groups, tumor growth inhibition ratio (C-T)/C% was 10.44%, 37.29%, and 44.04%, respectively. The inhibition ratio in 400 μg and 800 μg NJ001 group was statistically significant compared to the control group (Figure 6B). P=0.032, P=0.015). At the end of 3 weeks, the average tumor weight in the 200 μg and 800 μg NJ001 group was (1.51±0.20) g and (0.94±0.19) g, and the difference between the two treatments was also statistically significant (P=0.048).

Apoptosis of SPC-A1 Cells Induced by NJ001

As shown in Figure 7A, SPC-A1 cells in the NJ001 group exhibited a marginalized and condensed chromatin matrix, as well as shrinkage and blebbing of the cytoplasm and fragmented nuclei, which are typical features of apoptosis. In contrast, cells in either MCA2849 group or McAb free group maintained a normal morphology and retained an adequate ability to proliferate.

Compared to MCA2849 group and McAb free group, the high percentages of Annexin V+ cells (total apoptotic rate) in NJ001 group were observed at the time point of 24 h and 48 h (P<0.001 for both time points). The difference of total apoptotic rate in NJ001 group between 24 h time point and 48 h time point was also statistically significant (P=0.002, Figure 7B).

As shown in Figure 7C, the percentages of Annexin V+/PI+ cells (late apoptotic rate) in NJ001 group, MCA2849 group and McAb free group were 30.89%, 2.80% and 3.58% at 24 h time point respectively. The late apoptotic rate in NJ001 group was also higher than the other two groups at 48 h time point (P<0.001, P<0.001). Moreover, the late apoptotic rate in NJ001 group significantly increased after 24 h (from 24 h to 48 h time point) (P<0.001).

NJ001 induced the apoptosis of SPC-A1 cells in a time–dependant manner.

Discussion

Hybridoma technology is an available tool that potentially can produce anti-tumor antibodies and identify novel tumor antigens. In the past several years, considerable progress has been made in the identification of tumor-associated antigens recognized by McAbs or autoantibodies from patients. Currently, over 1,000 tumor-associated antigens have been reported [15–20].

In the present study, 3 McAbs were produced from 3 positive monoclonal hybridoma cell lines (NM001, NM004, NM005) that...
Figure 1. Production and characterization of NJ001. (A) Karyotype of NM001 hybridoma cell line (×400). (B) Binding activity of McAbs to human malignant and nonmalignant cells in culture. Undiluted supernatants from hybridomas NM001, NM004, NM005 were tested in triplicate with indirect cell ELISA as described in “Materials and Methods”. Each number represents the average absorbance of the substrate end product at 450 nm. Controls without McAbs exhibited an average absorbance of 0.02. NM001 was selected for further study as it exhibited the greatest binding ratio with lung tumor cells and the greatest specificity for the lung cancer cell lines compared with the other tumor cell lines. (C) Western blot analysis for the
reacted in varying degrees to lung cancer cells, normal cells, and the other cancer cell lines. McAb NJ001 was selected for further study as it exhibited the highest binding ratio with lung cancer cells and also exhibited the greatest specificity for the lung cancer cell lines as compared with the other cancer cell lines. The results of immunohistochemical staining indicated that NJ001 could positively react to NSCLC, but weak positively or negatively react to human small-cell lung cancer (SCLC), pulmonary pseudotumor and other epithelial tumors.

In addition to the high affinity and specificity, NJ001 also exhibited anti-tumor activity both \textit{in vitro} and \textit{in vivo}. We observed the effect of NJ001 on the proliferation of lung adenocarcinoma cell line SPC-A1. Results of soft agar assay showed that the colony formation efficiency in NJ001 groups reduced in a dose-dependent manner. The xenograft was established by subcutaneous injection of SPC-A1 cells and NJ001 was administered intraperitoneally at 3 different doses. NJ001 caused varying degrees of decrease in tumor volume and tumor weight compared with control mice. Moreover, when we injected the same amount of SPC-A1 cells cultured with NJ001 for 2 h in the same way, there was no tumor growth in nude mice. We also found that NJ001 induced the cytormorphological changes and significantly induced the apoptosis of SPC-A1 cells in a time-dependent mode. This suggested that the cell apoptosis induced by NJ001 is potentially the mechanism of the anti-tumor activity. Induction of apoptosis is mediated either through death receptors (an extrinsic pathway), or at the mitochondrial level (an intrinsic pathway) \cite{21-23}. Further identification of the apoptotic signaling pathways in SPC-A1 cells treated with NJ001 would be helpful in elucidating the mechanism by which NJ001 cause anti-tumor activity both \textit{in vitro} and \textit{in vivo}. Whereas, functional assays in our study were performed only on SPC-A1 cells used to generate NJ001. In the next study, we will do more work to observe the growth inhibitory effects of NJ001 extended beyond a single cell line and make it clear whether the biologic activity is specific to the cell line tested or represents a more generalized NSCLC response.

The importance of tumor antigens lies in their diagnostic and potential therapeutic utility \cite{24-33}. Additionally, tumor antigens can also provide prognostic information for the cancer patients \cite{34}. The tumor-associated antigens of human lung cancer have been recognized for many years; however, few reports have

**Figure 2. Photomicrographs of immunohistochemistry staining with NJ001 (×200).** Representative areas of tumor sections from (A) NSCLC lung adenocarcinoma; (B) NSCLC squamous lung cancer; (C) SCLC; (D) Breast carcinoma; (E) Gastric cancer; (F) Colon cancer; (G) Ovarian cancer; (H) Liver cancer; (I) Pulmonary pseudotumor; (J) Adjacent nontumourous lung tissues.

doi:10.1371/journal.pone.0033009.g002
investigated the common antigens or common epitopes of lung cancer [35,36]. In this study, the antigen which was finally named SP70 recognized by NJ001 was proven to be a protein with a Mr of 70 kDa. Visualization of NJ001 binding by indirect immunofluorescence indicated that SP70 was located in cytoplasm of SPC-A1. SP70 is a potential biomarker and therapeutic target for the immunotherapy of NSCLC.

In order to explore the function of NJ001 and the corresponding Ag, more work is needed to evaluate the clinical applicability. Furthermore, the marriage of target identification with antibody enhancement technologies will ultimately be translated into new and improved therapies for cancer patients, thus providing further support as to the importance of the continued study of NJ001 [7,37–39].

### Materials and Methods

#### Ethics Statement

This study was carried out in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiment of the First Affiliated Hospital of Nanjing Medical University (Permit Number: 19A5-2373). All efforts were made to minimize suffering.

Mononuclear cells (PBMC) from heparinized peripheral blood were recovered from healthy adult donors of the first affiliated hospital of Nanjing Medical University. For immunohistochemistry assay, NSCLC tissues (n = 106), SCLC tissues (n = 21), breast carcinoma tissues (n = 21), gastric cancer tissues (n = 5), colon cancer tissues (n = 5), ovarian cancer tissues (n = 5), liver cancer tissues (n = 5), pulmonary pseudotumor tissues (n = 25) and adjacent non tumourous lung tissues (n = 8) were obtained from the department of pathology in the same hospital between July 2009 and June 2010.

This study was approved by the Committee on the Ethics of Treatment of Human Subjects of the First Affiliated Hospital of Nanjing Medical University, and a written informed consent was also obtained from each participant.

### Cells and Cell Lines

Ten different human cell lines or cultures (listed in Figure 1B) representing various normal and neoplastic tissues were used to characterize the antibodies in this study. All of the cell lines were purchased from cell bank of the Chinese Academy of Sciences in Shanghai. Human lung cancer cell lines SPC-A1, NCI-H520, NCI-H460, and NCI-H446, colon carcinoma cell line COLO 205 and normal fetal lung cell line WI-38 were grown in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco Invitrogen, Carlsbad, CA). Human lung cancer cell lines A549, breast carcinoma cell line ZR-75-30, liver carcinoma cell line HepG2 were cultured in RPMI1640
supplemented with 10% (v/v) FBS. Mononuclear cells (PBMC) from heparinized peripheral blood were recovered from healthy adult donors (from the first affiliated hospital of Nanjing Medical University) by Ficoll-Hypaque density gradient centrifugation.

Generation of McAb

Monoclonal antibodies were produced as follows. 6–8 week old female BALB/c mice were intraperitoneally immunized with $1 \times 10^6$ SPC-A1 cells three times over a 3–4 week interval, then spleen cells were hybridized with SP2/0 (BALB/c mice myeloma cell line) in 50% PEG-4000 (Sigma, USA) grown in HAT medium in RPMI1640 (GIBCO, USA). Culture supernatants were screened for antibody reactivity to WI-38 and SPC-A1 cells using a live cell, solid-phase ELISA. Target cells ($1 \times 10^5$) were mixed with 0.3% agarose and different concentrations of NJ001 or MCA2849 were layered on the top of culture media in 6-well culture plates and allowed to grow for 2 weeks before colonies were counted.

Table 2. Results of colony formation of SPC-A1 cells treated by NJ001 or MCA2849 in soft agar.

| NJ001 | MCA2849 |
|-------|---------|
| Concentration of NJ001 (μg/mL) | Average number of colonies (≥50 cells/colony) | Colony formation efficiency (%) | Inhibition ratio of colony (%) | Concentration of MCA2849 (μg/mL) | Average number of colonies (≥50 cells/colony) | Colony formation efficiency (%) | Inhibition ratio of colony (%) |
| 0 | 192 ± 7.07 | 0.96 | 0 | 202 ± 9.71 | 1.01 | 0 | 2 |
| 100 | 147 ± 12.73 | 0.735 | 23.4 | 100 | 191 ± 7.63 | 0.96 | 5.45 |
| 200 | 72 ± 4.24 | 0.36 | 62.5 | 200 | 182 ± 6.08 | 0.91 | 9.90 |
| 400 | 0 | 0 | 100 | 400 | 183 ± 5.77 | 0.92 | 9.41 |
| 800 | 0 | 0 | 100 | 800 | 186 ± 10.0 | 0.93 | 7.92 |
| 1000 | 0 | 0 | 100 | 1000 | 183 ± 11.26 | 0.92 | 9.41 |

*Colony formation efficiency = (average number of colonies/average number of cells added per well) × 100%.

**Inhibition ratio of colony formation = (1 - average number of colonies in NJ001 or MCA2849 group/average number of colonies in McAb free group) × 100%.

Figure 4. Inhibition of colony formation of SPC-A1 cells by NJ001 in soft agar (×1000). The cell suspensions (2 × 10^6 cells) mixed with 0.3% agarose and different concentrations of NJ001 or MCA2849 were layered on the top of culture media in 6-well culture plates and were incubated in a CO2 incubator. Representative contrast images were shown: (A) 0 μg/mL NJ001, (B) 100 μg/mL NJ001, (C) 200 μg/mL NJ001, (D) 400 μg/mL NJ001, (E) 100 μg/mL MCA2849, (F) 200 μg/mL MCA2849, (G) 400 μg/mL MCA2849, (H) 800 μg/mL MCA2849.
first plated in 96 wells and incubated for 18 h to 24 h at 37°C in 5% CO₂. The growth medium was then aspirated and the cells were fixed for 15 min at room temperature with 95% ethanol. The cell membranes were broken in triton X-100 for 20 min and the cells were then blocked with 5% BSA for 60 min at room temperature.

The positive hybridoma cells were subcloned using a limiting dilution. Monoclonal hybridoma cells with a high valence against SPC-A1 cells were expanded and retransfused into the abdominal cavity of the BALB/c mice to prepare the ascites. The McAbs were further purified from the ascites via Protein A affinity chromatography.

The positive hybridoma cell were treated with colchicine. After 10% Giemsa dyeing, we observed mid-term nuclear cells and analyzed the karyotype by the microscope.

**Characterization of McAbs**

The heavy and light chain composition of 3 McAbs were determined using the ISO Strip Kit (Santa Cruz Biotechnology, Inc, USA). The extent of McAbs binding to various normal and malignant cells (listed in Figure 1B) were determined with 0.1 mL of culture supernatant from positive hybridoma cells and 1×10⁴ target cells using ELISA for the screening described above, and the production of substrate was measured spectrophotometrically at 450 nm.

**Indirect Immunofluorescence Analysis**

Indirect immunofluorescence was performed as follows. Briefly, cells were grown to 80% confluence on cover slips and then fixed in 95% ethanol at room temperature. After washing with PBS, the cell membranes were broken in triton X-100 for 20 min and blocked with 5% BSA for 60 min at room temperature, and this was followed by incubation with FITC conjugated goat-anti-mouse IgG (1:300) for 45 min at room temperature. The slides were counterstained with Hoechst. Immunofluorescence staining results were obtained using fluorescence and confocal microscopy (Zeiss LSM 710, Germany).

**Western Blot Analysis**

In order to perform the Western blot analysis, the nine cell lines were initially cracked by the RIPA lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 50 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/mL Aprotinin, 1 mM Na₃VO₄, 1 mM NaF). Next, 25 μg of total protein from the nine cell lines was electrophoresed on a 12% SDS-PAGE gel, and then transferred to a PVDF membrane (Amersham Pharmacia Life Science, USA). After blocking with 10% non-fat milk in TBST, the PVDF membrane was incubated with NJ001 (1:300) and a anti-GAPDH antibody (Zhongshan Biological, Beijing, China) over night at 4°C to confirm equivalent protein loading in each lane. This was followed by incubation with HRP-conjugated goat-anti-mouse IgG for 1 h at room temperature. The PVDF membrane was further washed 3 times with TBST for 15 min each, and finally developed with ECL (Amersham Life Science) on X-ray film.

**Immunohistochemistry**

NSCLC tissues (n = 106), SCLC tissues (n = 21), breast carcinoma tissues (n = 21), gastric cancer tissues (n = 5), colon cancer tissues (n = 5), ovarian cancer tissues (n = 5), liver cancer tissues (n = 5), pulmonary pseudotumor tissues (n = 25) and adjacent nontumourous lung tissues (n = 8) were obtained from the department of pathology in the first affiliated hospital of Nanjing Medical University. NSCLC tissues included lung adenocarcinoma tissues (n = 58) and squamous lung cancer tissues (n = 48). Tissue sections were treated with 0.3% hydrogen peroxidase for 5 min, followed by 30 min blocking with normal goat serum at room temperature. NM001 (1:200) was applied to the blocked sections and incubated overnight at 4°C. The sections were incubated for 30 min at 37°C with HRP-labeled goat-anti-mouse IgG antibody (1:2,000), and the positive signals were visualized by development in diaminobenzidine tetrahydrochloride (DAB) solution. The sections were viewed under an Olympus Ax-70 DMC Ie CCD camera connected to a PC monitor.

**Cell Proliferation Assay**

SPC-A1 cells were seeded in a 96-well plate at 5,000 cells per well. Culture supernatants from positive hybridoma cells and SP2/0 cells were added. During the final 16 h of the 24 h, 48 h, and 72 h incubation at 37°C, the cells were pulsed with 0.5 μCi/well [³H] thymidine. Proliferation assays were performed by liquid scintillation counting of the harvested cells. Results of SPC-A1 cell proliferation measurement were presented as the count per minute (cpm).

**Soft Agar Assay**

 Colony formation was analyzed by soft agar assay, using the anchorage-dependent, lung adenocarcinoma cell line SPC-A1 as a tumor cell model, according to the procedures of Hong KW [40].

---

**Figure 5. Photomicrographs of H&E staining in the preliminary study (×200).** Representative areas of tissue sections from inoculation sites in NJ001 group (A) and the excised tumors in control group (B). doi:10.1371/journal.pone.0033009.g005
briefly, a bottom layer of 0.5% agarose (Promega, U.S.A) containing of 2 mL culture medium was initially solidified in a
6-well culture plate. Next, 2 mL of 0.5% agarose solution containing 2×10^6 cells with different concentrations of NJ001 or
MCA2849 (irrelevant McAb) (0, 100, 200, 400, 800, or 1000 μg/mL) was layered on top. Each dose was tested in triplicate. After
incubation at 37°C with 5% CO2 atmosphere for 2 weeks, the colonies that contained over 50 cells were counted under a
microscope. The colony formation efficiency and the inhibition ratio of the colony were calculated as the following formulas:
colony formation efficiency = (average number of colonies/average number of cells added per well)×100%; inhibition ratio of
colony formation = (1- average number of colonies in NJ001 group/average number of colonies in McAb free group)×100%.
This experiment was repeated 3 times.
Anti-tetanus McAb (MCA2849) (AbD Serotec, Germany) was used as irrelevant McAb in this study.

Xenograft Experiment
Thirty female BALB/c nude mice of 6-week old were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai,
China). The SPC-A1 cells were maintained in 10% FBS RPMI1640 medium until the cells reached 90% confluence. All
procedures were conducted in accordance to the Animal Care and Use Committee guidelines of Nanjing Medical University.

In the preliminary study, SPC-A1 cells were incubated with NJ001 at 37°C in a 5% CO2 incubator for 2 h. Thus each 200 μL
saline contained 2×10^6 SPC-A1 cells and 400 μg NJ001. The lateral axilla of 5 nude mice were inoculated subcutaneously with
the solution, and the control group, also composed of 5 mice, was injected the equivalent SPC-A1 cells in the same position. After
three weeks of inoculation, mice were euthanized and the tissues were obtained for H&E staining.

The mice were randomly divided into 4 groups, with 5 mice per group. The xenograft was established by subcutaneous injection
of 2×10^6 SPC-A1 cells/200 μL per mouse into the lateral axilla. Antibodies were administered intraperitoneally at 3
different doses (200 μg, 400 μg, or 800 μg per mouse). Treatment was initiated simultaneously with the implantation and consisted
of two steps: daily injection for the first week, followed by injections twice a week for the proceeding two weeks. The control
group received sterile saline injections in the same mode. Animals were monitored for tumor size at 4-day intervals. The tumor
volumes (mm^3) was calculated according to the following equation: Volume = width^2×length/2 [28,41]. All mice were euthanized
three weeks after the initiation of treatment and tumors were removed and weighed. Tumor growth inhibition was calculated
by the formula: tumor growth inhibition ratio = (1 – average tumor weight in NJ001 group/average tumor weight in control
group)×100%. Treatment toxicity was assessed by the physical appearance of the animals.

H&E Staining
Tissues obtained were fixed in 10% formalin and embedded in paraffin. The embedded tissues were subsequently cut into 4 μm
sections and placed on glass slides for H&E staining.

Apoptosis Assay
SPC-A1 cells were seeded in a 12-well plate at 1×10^5 cells per well. After overnight incubation, SPC-A1 cells were cultured
with or without 200 μg/mL NJ001 or MCA2849 for 24 h and 48 h. Each time point was tested in triplicate. The morphological
changes of the cells were then observed and the rate of apoptosis was determined by flow cytometry. Briefly, cells were
collected, washed with PBS and resuspended in 500 μL binding buffer containing 10 mmol/L HEPES-NaOH (pH 7.4),
140 mmol/L NaCl, and 2.5 mmol/L CaCl2. Next, 5 μL of Annexin V-FTTC (Bender MedSystems, Austria) and 5 μL of
propidium iodide (PI) solution (Bender) were added and the cells were then incubated in the dark for 15 min. The fluorescence
was then analyzed by flow cytometry. Early apoptosis and late apoptosis was determined as the percentage of Annexin V+/PI-
cells and Annexin V+/PI- cells, respectively. The rate of total apoptosis was the sum of early and late apoptosis. This
experiment was repeated 3 times.

Statistical Analysis
All values were expressed as mean ± standard deviation. Mean comparison of groups was conducted using single factor variance
analysis. The pairwise comparison was performed with the LSD test if the variance was homogeneous and a P-value < 0.05 was
regarded as statistically significant, while Dunnett’s C method was used for the heterogeneity of variance, setting 0.05 as the
significance level.
McAb NJ001 against NSCLC and Its Characteristics

A

B

C

24 h NJ001

24 h MCA2849

24 h McAb free

48 h NJ001

48 h MCA2849

48 h McAb free

Annexin V-FITC

Annexin V-FITC

PI

PI

An

Annexin V+ cells (%)

An

Annexin V+ cells (%)

24 h

48 h

**
Figure 7. NJ001 induced apoptosis of SPC-A1 cells. SPC-A1 cells were cultured with or without 200 μg/mL NJ001 or MCA2849 for 24 and 48 h. (A) Morphological changes in SPC-A1 cells were observed under inverted microscope (>100). a, 24 h NJ001; b, 24 h MCA2849; c, 24 h McAb free; d, 48 h NJ001; e, 48 h MCA2849; f, 48 h McAb free. (B) Apoptosis was analyzed by flow cytometry. (C) Each column and error bar represents the mean ± SD of three independent experiments (**P<0.001). The amount of late apoptosis was determined as the percentage of Annexin V+/PI+ cells.
doi:10.1371/journal.pone.0033009.g007

Acknowledgments
We are grateful to the technical support from National Key Clinical Department of Laboratory Medicine of China in Nanjing.

References
1. Jermal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60: 27–50.
2. Molina JR, Yang P, Casiavi SD, Schädel SE, Adjei AA (2008) Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 83: 584–594.
3. Jermal A, Than MJ, Ries LA, Howe HI, Wei HR, et al. (2008) Annual report to the nation on the status of cancer, 1975–2005, featuring trends in lung cancer, tobacco use, and tobacco control. J Natl Cancer Inst 100: 1672–1694.
4. Kunitoh H, Suzuki K (2005) How to evaluate the risk/benefit of trimodality therapy in locally advanced non-small-cell lung cancer. Br J Cancer 96: 1490–1503.
5. Hanna N, Shepherd FA, Fossella FV, Pereira JR, De Marinis F, et al. (2005) An alternative strategy for the management of non-small-cell lung cancer: Hematol Oncol Clin North Am 19: 169–208.
6. Vansteenkiste JF, Sroshchts S (2004) Positron emission tomography in the management of non-small-cell lung cancer. Hematol Oncol Clin North Am 18: 269–288.
7. Reichert JM, Valge-Archer VE (2007) Development trends for monoclonal antibodies produced by mAb proteomics. J Proteome Res 9: 1834–1842.
8. Antigen identification and characterization of lung cancer specific monoclonal antibodies. J Proteome Res 6: 427–437.
9. Application of phage display to high throughput antibody generation and characterization. Genome Biol 8: 1–18.
10. Pal SK, Fuglin RA, Reckamp K (2009) Targeted therapies for non-small cell lung cancer: an evolving landscape. Mol Cancer Ther 9: 1931–1944.
11. Reck M, Crino L (2009) Advances in anti-VEGF and anti-EGFR therapy for advanced non-small cell lung cancer. Lung Cancer 63: 1–9.
12. Gridelli C, Maiseon P, Ferrara ML, Rosi A (2009) Cetuximab and other anti-epidermal growth factor receptor monoclonal antibodies in the treatment of non-small cell lung cancer. Oncologist 14: 603–611.
13. Collmannsberger G, Schittenhelm M, Honecker F, Tillner J, Weber D, et al. (2005) A phase I study of the humanized monoclonal anti-epidermal growth factor receptor (EGFR) antibody EMD 7200 (matuzumab) in combination with paclitaxel in patients with EGFR-positive advanced non-small-cell lung cancer (NSCLC). Ann Oncol 17: 1007–1013.
14. Krug LM, Miller VA, Patel J, Crapanzano J, Azoli CG, et al. (2005) Randomized phase II study of weekly docetaxel plus trastuzumab versus weekly paclitaxel plus trastuzumab in patients with previously untreated advanced non-small cell lung carcinoma. Cancer 104: 2149–2155.
15. Ding Y, Wang Y, Li Y, Hou Y, Peng D, et al. (2006) An alternative strategy for high throughput generation and characterization of monoclonal antibodies against human plasma proteins using fractionated native proteins as immuno-gens. Proteomics 6: 439–446.
16. Gao J, Guo Y, Ju Y, Yang J, Wu Q, et al. (2006) Proteomics-based generation and characterization of monoclonal antibodies against human liver mitochondrrial proteins. Proteomics 6: 427–437.
17. Saito S, Murayama Y, Tani T, Fujimura T, Taima T, et al. (2005) Haptoglobin-beta chain defined by monoclonal antibody RM2 as a novel serum marker for prostate cancer. Int J Cancer 123: 633–640.
18. Schofield D, Pope A, Clementel V, Buckell J, Chapple S, et al. (2007) Application of phage display to high throughput antibody generation and characterization. Genome Biol 8: 1–18.
19. Lu ZJ, Song QF, Jiang SS, Song Q, Wang W, et al. (2009) Identification of ATP synthase beta subunit (ATP8B1) on the cell surface as a non-small cell lung cancer (NSCLC) associated antigen. BMC Cancer 14: 9–16.
20. Wang D, Hincapie M, Guergovs-Kuras M, Kardas J, Takacs L, et al. (2010) Antigen identification and characterization of lung cancer specific monoclonal antibodies produced by an mAb proteomics. J Proteome Res 9: 1834–1842.

Author Contributions
Conceived and designed the experiments: SYP FW PJH. Performed the experiments: SYP FW PJH TX LXZ JX QL WYX RHS LH YP XJQ. Analyzed the data: SYP FW PJH YQS ZBH HBS. Contributed reagents/materials/analysis tools: SYP ZBH HBS. Wrote the paper: SYP FW PJH TX.