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CD44 promotes cell proliferation in non-small cell lung cancer

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Abstract. Cluster of differentiation 44 (CD44), a marker for cancer stem cells, has been reported to be associated with poor prognosis in non-small cell lung cancer (NSCLC), but its involvement in tumor growth has not fully been elucidated. The present study explored the associations between CD44 expression and clinicopathological features using immunohistochemistry in 94 NSCLC cases, as well as proliferation in cells with aberrant expression of this protein. Overexpression of CD44 was achieved by transfecting H1299 cells with CD44 expression vectors, and inhibition of CD44 expression was performed by transfecting small interfering RNAs into A549 cells. Cell proliferation was measured using Cell Counting Kit-8 assays and flat plate colony formation assay was performed to confirm the cellular anchorage growth *in vitro*. In total, 64 (68.1%) of the 94 NSCLC cases stained positive for CD44 expression. High expression of CD44 was associated with advanced T stage in NSCLC. Overexpression of CD44 in H1299 cells promoted cell proliferation and colony formation *in vitro*. Meanwhile, knockdown of CD44 expression in A549 cells suppressed cell proliferation and colony formation *in vitro*. High expression of CD44 may promote NSCLC progression by increasing cancer cell proliferation; therefore, it may serve as a potential biomarker for diagnosis or target therapy.

Introduction

At present, lung cancer is the leading cause of cancer-associated mortality worldwide in men and women (1). Despite the discovery and application of new standard therapies, the current 5-year relative survival rate for lung cancer remains at 18% (1). Lung cancer is classified into two major categories according to pathological type: Non-small cell lung cancer (NSCLC) and small cell carcinoma. NSCLC, the most common type of lung cancer, accounts for ~80-85% of all lung cancer cases (2). The disease stage at diagnosis, including the T stage, has been reported to predict the prognosis of NSCLC (3). However, the effects of gene expression on tumor growth in NSCLC remain far from being completely understood.

CD44, a 85-90 kDa transmembrane glycoprotein, is widely expressed on the surface of cells in the majority of normal and carcinomatous human tissues (4). In normal human cells, CD44 has been reported to be involved in multitudinous cellular functions, including proliferation, adhesion, migration, hematopoiesis, lymphocyte activation, homing and extravasation (5). In various types of human malignancy, high levels of CD44 expression have been demonstrated to be associated with cancer progression (6-10). In lung cancer, diverse studies have clarified that the expression of CD44 is associated with tumorigenesis and malignant features of NSCLC (11,12). Tumor and serum expression of CD44 have been confirmed as prognostic indicators for patients with NSCLC (13,14). In addition, overexpression of CD44 is associated with the occurrence and migration of NSCLC (15). Furthermore, accumulating evidence has demonstrated that lung cancer cells expressing CD44 tend to have stem cell-like properties (16-18) and that cancer stem cells are responsible for driving metastasis (16). CD44 also contributes to drug resistance in lung cancer (19). Although evidence demonstrating that CD44 may act as an oncogene in NSCLC exists, direct cell experiments are still required to further confirm the effect of CD44 on the malignant phenotype of lung cancer cells.

The present study aimed to provide more evidence for the involvement of CD44 in the progression of lung cancer, and so evaluated the clinical significance of CD44 expression in patients with NSCLC and investigated the effect of CD44 on the proliferation of NSCLC cells.

Materials and methods

Cell lines. NSCLC cell lines, including H1975, H1299, H520, A549, PC-9 and GLC82, were obtained from American Type Culture Collection (Manassas, VA, USA). The H520 cell line

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was derived from lung squamous cell carcinoma, while the other 5 cell lines were all derived from adenocarcinoma. Among the 5 lung adenocarcinoma cell lines, H1650 was derived from bronchoalveolar carcinoma cells from the pleural effusion of a Caucasian male; H1299 was derived from the lymph nodes of a male patient with lung adenocarcinoma; A549 was also derived from a Caucasian male with lung adenocarcinoma; PC9 was established by Tokyo University (Tokyo, Japan) and derived from adenocarcinoma in pleural effusion; and GLC82 was derived from a female Chinese patient with lung adenocarcinoma.

Cell culture, antibodies, and reagents. All 6 cell lines were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C with 5% CO_2 in a humidified atmosphere. Monoclonal mouse anti-human CD44 antibodies were purchased from Abcam (cat no. ab6124; Cambridge, UK).

Tissue specimens and immunohistochemistry. There were 94 cases of NSCLC tissue specimens excluding adjacent normal tissues acquired from patients who underwent surgery at Peking University Cancer Hospital and Institute (Beijing, China) from July 2013 to October 2015 without any other treatments prior to surgery. Clinicopathological characteristics of these patients are presented in Table I with 72 cases of squamous carcinoma, 19 cases of adenocarcinoma, 2 cases of adenosquamous carcinoma and one carcinoid. Written informed consent was obtained from all patients included in the present study, and this study was approved by the Ethics Committee of Peking University Cancer Hospital and Institute.

Tumor tissues were fixed with 10% formaldehyde solution overnight at room temperature and then paraffin-embedded tumors were cut into 4-μm thick sections. Sections were routinely stained, as described in our previous study (20). Briefly, the sections were immersed in xylene to remove the paraffin, washed in a graded series of ethanol, immersed in citrate buffer and then incubated in a high-pressure sterilization oven for antigen retrieval with citrate buffer at pH 6.0 for 3 min at 100˚C. Endogenous peroxidase activity was blocked in a blocking solution with 3% H_2O_2 in PBS for 10 min at room temperature, and then the sections were incubated with PBS containing 1% bovine serum albumin (Amresco, Solon, OH, USA) for 10 min at room temperature to block non-specific binding. Following washing in PBS, the tissue sections were incubated at room temperature for 1 h with antibodies against CD44 (cat no. ab6124; dilution, 1:2,000; Abcam), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (cat no. A4416; 1:1,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature. Then, the slides were visualized with 0.1% 3,3-diaminobenzidine (Sigma-Aldrich; Merck KGaA) for 2 min, and counterstained with one drop of 1% hematoxylin for 10 min at room temperature. According to the percentage of cells with complete membrane staining in the tumor tissue section, the section was classified as follows: Negative, <15%; weak positive, 15-30%; moderate positive, 30-75%; and strong positive, 75-95%. In the present study, CD44 expression was either defined as negative, or positive (either weak, moderate, or strong positive).

Plasmid construction and cell transfection. Human CD44 open reading frame mammalian expression plasmids were purchased from YouBio (Chongqing, China, http://www.youbio.cn/). H1299 cells were seeded onto 6-well plates at 80% confluence, and then transfected with 4.0 μg of CD44 plasmids or pcDNA3.0 empty plasmids (Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. After 24 h, cells were cultured and propagated in media containing 500 μg/ml G418 (Sigma-Aldrich; Merck KGaA) at 37˚C with an atmosphere containing 5% CO_2 in a humidified incubator until a stable cell line was established. Small interfering RNAs (siRNAs) targeting CD44 were designed using BLOCK-iT™ RNAi Designer (Thermo Fisher Scientific, Inc.) within open reading frame, with the default criteria on the webpage (http://rnaidesigner.thermofisher.com/rnaiexpress), and then synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). siRNAs (50 nmol/l) were transfected into A549 cells using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The CD44 siRNA target sequences were as follows: siRNA-1, 5'-CUCCCAGUAUGACACAUAUTT-3'; siRNA-2, 5'- GGACCAAUUACCAUAACUATT-3'; siRNA-3, 5'-GCAGUCAACAGUCGAAGAATT-3'; and NC, 5'-UUCUCCGAACGUGUCACGUTT-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. 1 μg total RNA was reverse transcribed using TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for PCR (TransGene, Beijing, China, http://www.transgen.com.cn), according to the manufacturer's protocol. qPCR reactions were performed using a Light-Cycler 480 Real-Time PCR System (Roche Diagnostics GmbH) and the LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics GmbH). GAPDH was used as the internal control. The primers sequences are as follows: CD44, forward 5'-ACACGAAGGAAAGCAGGACC-3', and reverse 5'-TTTGCTCCACCTTCTTGACTC-3'; GAPDH, forward 5'-TGAAGGTCGGAGTCAACGG-3', and reverse 5'-CTGGAAGATGGTGATGGGATT-3'. The cycling conditions were as follows: 5 min at 95˚C, followed by 42 cycles of 10 sec at 95˚C, 30 sec at 60˚C, and 20 sec at 72˚C. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Cq}$ method (where $\Delta Cq=Cq_{target}-Cq_{control}$) (21).

Western blot. Cells were lysed in RIPA buffer (Roche Diagnostics GmbH) containing a complete protease inhibitor cocktail for 15 min on ice. The bicinchoninic acid assay method (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to determinate protein quantity and equivalent amounts of total proteins (30 μg) were separated by 10% SDS-PAGE and transferred topolyvinylidene fluoride membranes (Millipore, Billerica, MA, America). Membranes

Table I. Associations between CD44 expression and clinicopathological features in non-small cell lung cancer cases.

	CD44 expression				
Variable	n (+)	(%)	n (-)	(%)	P-value
Age, years					0.532
<60	30	71.4	12	28.6	
≥60	34	65.4	18	34.6	
Sex					1.000
Male	32	68.1	15	31.9	
Female	32	68.1	15	31.9	
Smoking					0.791
Yes	28	66.7	14	33.3	
No	36	69.2	16	30.8	
Pathology					0.211
Squamous carcinoma	51	70.8	21	29.2	
Adenocarcinoma	10	52.6	9	47.4	
Adenosquamous carcinoma	2	100.0	0	0.0	
Carcinoid	1	100.0	0	0.0	
T-stage					0.032
T1	33	58.9	23	41.1	
T2	28	80.0	7	20.0	
T3	3	100.0	0	0.0	
N stage					0.917
N0	37	68.5	17	31.5	
N1/2/3	27	67.5	13	32.5	
TNM stage					0.279
I	34	70.8	14	29.2	
II	12	54.5	10	45.5	
III	18	75.0	6	25.0	

CD44, cluster of differentiation 44; TNM, tumor-node-metastasis.

were blocked with 5% nonfat dried milk dissolved in TBST containing 0.1% Tween 20 for 1 h at room temperature and then incubated with following specified antibodies for 1 h at room temperature: Anti-CD44 (cat no. ab6124; dilution, 1:5,000; Abcam) and mouse anti-GAPDH (cat no. 97166; dilution, 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA), followed by HRP-conjugated goat anti-mouse IgG (cat no. A4416; dilution, 1:5,000; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Signals were visualized using an enhanced ECL Chemiluminescence reagent (cat no. WBKLS0500; Merck KGaA) and detected using AI600 version 1.2.0 on Amersham Imager 600 (GE Healthcare, Chicago, IL, USA).

Flow cytometry. Briefly, a total of 10,000 live cells were incubated with anti-CD44 (cat no. ab6124; dilution, 1:100; Abcam) at 37˚C for 30 min. Following washing with PBS, cells were then incubated with FITC-labeled goat anti-mouse IgG (cat no. F0257; dilution, 1:100; Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min. Following washing three times with PBS, the fluorescence intensity was detected with the BD Accuri™ C6 Flow Cytometer with BD Accuri C6 software version 1.0.264.21 (BD Bioscience, San Diego, CA, USA).

Cell proliferation assay. Cells were seeded into 96-well plates (1,500 cells/well). To assess cell proliferation, cells were subjected to Cell Counting Kit-8 (CCK-8) assays (Dojindo, Kumamoto, Japan) at 0, 12, 24, 36, 48, 60 and 72 h. Each time, supernatant was replaced by RPMI-1640 medium containing 10% CCK-8 reagent. After 2 h incubation at 37˚C, the absorbance at 450 nm was measured to determine the number of viable cells, according to the manufacturer's protocol. The experiment was repeated three times independently. The cell doubling time (DT) was calculated by the equation: TD=ΔTx[lg2/(lgNt-lgN0)], where ΔT is the time interval; N0 is the initial cell number and Nt is the end point cell number.

Clonogenic assay. A total of ~500 cells were seeded into 60-mm dishes, and the culture medium of RPMI-1640 medium supplemented with 10% FBS was changed every 3 days. Visible colonies were fixed after 8 days in 4% paraformaldehyde for 15 min at room temperature, stained with 0.1% crystal violet solution for 30 min at room temperature, and then washed with PBS. The number of colonies number was counted by naked eye from three independent experiments.

Statistical analysis. All statistical analysis was performed using SPSS 22.0 software (IBM Corp, Armonk, NY, US). Associations between CD44 expression and clinicopathological factors were analyzed using Pearson's χ^2 test and Fisher's exact tests. The mRNA levels, colony number and size were presented as the mean ± standard deviation from three independent experiments. The difference between two groups was assessed using independent samples t-test. Dunnett's Multiple Comparison tests were used to compare differences between treatment groups and the control group following analysis of variance (ANOVA). Two-way ANOVA tests were used to analyze the difference of cell viability curves for two groups. $P<0.05$ (two-tailed) was considered to indicate a statistically significant difference.

Results

NSCLC tissues express CD44. As presented in Fig. 1, CD44 was predominantly expressed in the cell membrane of NSCLC cells. CD44 expression was observed in all pathological types of NSCLC tissue, including squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma and carcinoid. The percentage of CD44-stained tumor cells in the samples ranged from 0-95%. To facilitate analysis, CD44 expression was classified into two groups; negative and positive. When >15% of tumor cells stained positively for CD44 in a sample, it was classified as positive CD44 expression; otherwise it was defined as negative. In total, 64 (68.1%) of 94 NSCLC cases were identified as positive for CD44 expression.

High levels of CD44 expression are associated with an advanced T stage. To predict the clinical significance of CD44 expression, we analyzed the associations between CD44 expression and clinicopathological features in NSCLC. There was no significant difference between CD44 expression and

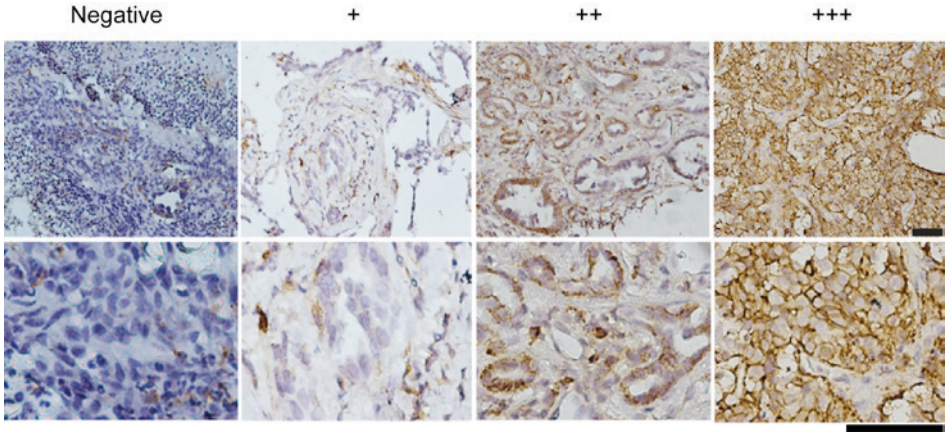

Figure 1. Expression of cluster of differentiation 44 in non-small cell lung cancer tissues was assessed using immunohistochemistry. Scale bar: 100 μm.

age, sex, smoking status, pathology type, N classification or TNM stage (22). However, the level of CD44 expression was positively associated with an advanced T classification ($P<0.05$; Table I). A total of 58.9% of cases (33 of 56) stained positive for CD44 in the T1 group, while 80.0% of cases (28 of 35) stained positive for CD44 in the T2 group, and all cases (3 of 3) stained positive for CD44 in the T3 group. This result suggested that the higher the T stage was, the stronger the CD44 staining in the tumor tissue would be, indicating that CD44 potentially promotes tumor growth.

High levels of CD44 expression are positively associated with cell proliferation in NSCLC cell lines. To further investigate the relationship between CD44 expression and cell proliferation, CD44 expression (Fig. 2A) and cell doubling time (Fig. 2B) were determined in NSCLC cell lines. Among these cells, A549 cells and CLC82 cells had relative high CD44 expression, while PC9, H520 and H1299 had the relative low CD44 expression. Thus, H1299 were selected to perform the CD44 over-expression experiments and A549 cells to do the CD44 interference experiments. The data from the present study suggested that A549 cells had the highest CD44 expression and the shortest doubling time of the 6 NSCLC cell lines (Fig. 2A and B). The line graph in Fig. 2C also suggested a positive association between CD44 expression and cell doubling time, indicating that higher CD44 expression tended to lead to faster cell proliferation.

Overexpression of CD44 promotes H1299 cell proliferation. To investigate the forced expression of CD44 on cell malignancy, we forced expression of CD44 in H1299 cells with a low endogenous CD44 expression (Fig. 2A). This was conducted using plasmid transfection and G418 screening, a pool of H1299 cells that stably overexpressed CD44, named H1299-CD44, were established, and the negative control cells transfected with empty vectors were named H1299-control. CD44 mRNA and protein expression of the two cell lines by RT-qPCR (Fig. 3A) and western blotting (Fig. 3B). As presented in Fig. 3A, the expression of CD44 increased ~17.4 fold in H1299-CD44 cells compared with H1299-control cells. In addition, the protein expression of CD44 also visibly increased in H1299-CD44 cells compared with H1299-control cells (Fig. 3B; lane 2), indicating a successful CD44 overexpression in H1299-CD44

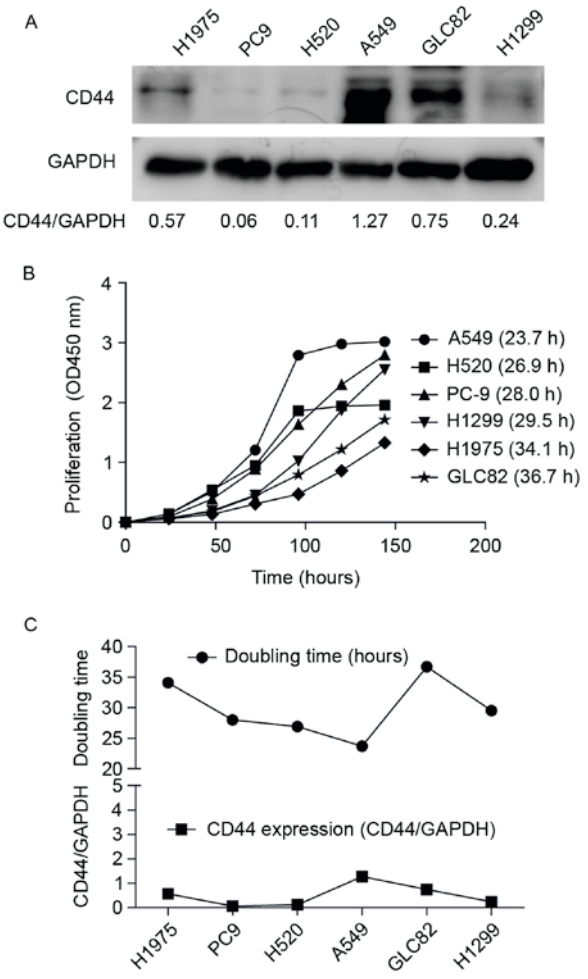

Figure 2. Expression of CD44 in NSCLC cell lines. (A) Western blot analysis of CD44 in 6 NSCLC cell lines. (B) Cell Counting Kit-8 assay analysis of the doubling time the 6 NSCLC cell lines. (C) Line graph analysis of the association between CD44 expression and cell doubling time of the 6 NSCLC cell lines. CD44, cluster of differentiation 44; NSCLC, non-small cell lung cancer.

cells. Consistent with this result, CCK-8 assays suggested that H1299-CD44 cells proliferated significantly faster than the negative control cells (Fig. 3C; $P<0.001$). The difference between H1299-CD44 cells and H1299-control cells became

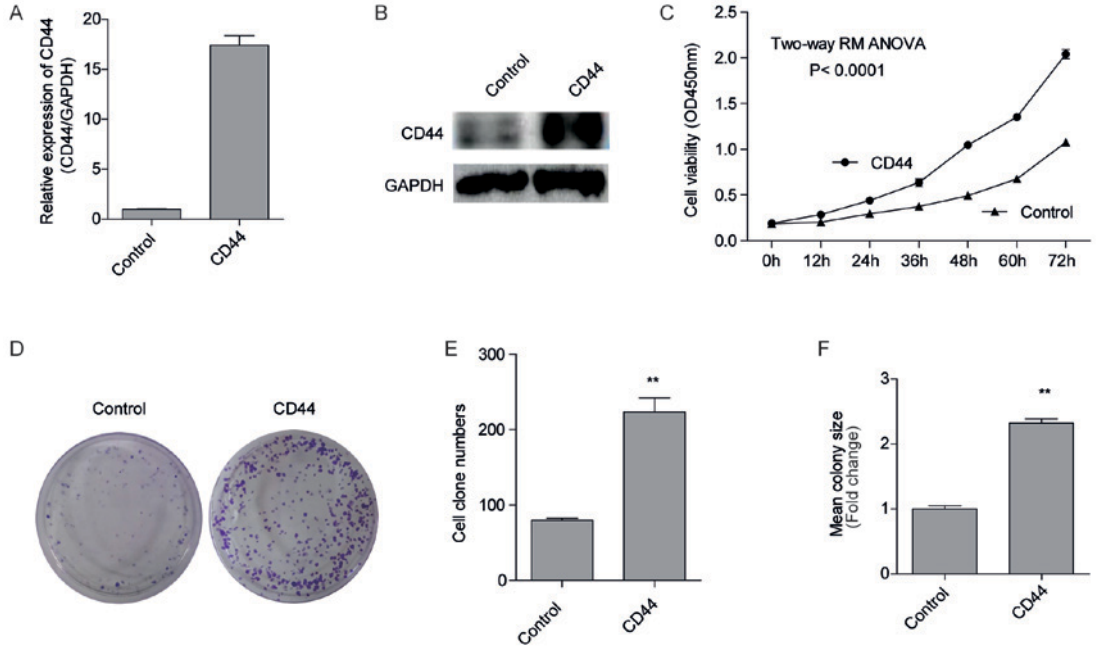

Figure 3. Effect of CD44 overexpression in H1299 cells. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis of CD44 in H1299-control and H1299-CD44 cells. (C) Cell Counting Kit-8 of H1299-control and H1299-CD44 cell proliferation. (D) Plate colony formation assay analysis of the proliferation of H1299-control and H1299-CD44 cells. Quantification of (E) the colony number and (F) colony size of the H1299-control and H1299-CD44 cells. Bars and error bars indicate the mean and standard deviation, respectively, from three different experiments. **P<0.01 vs. H1299-control. CD44, cluster of differentiation 44.

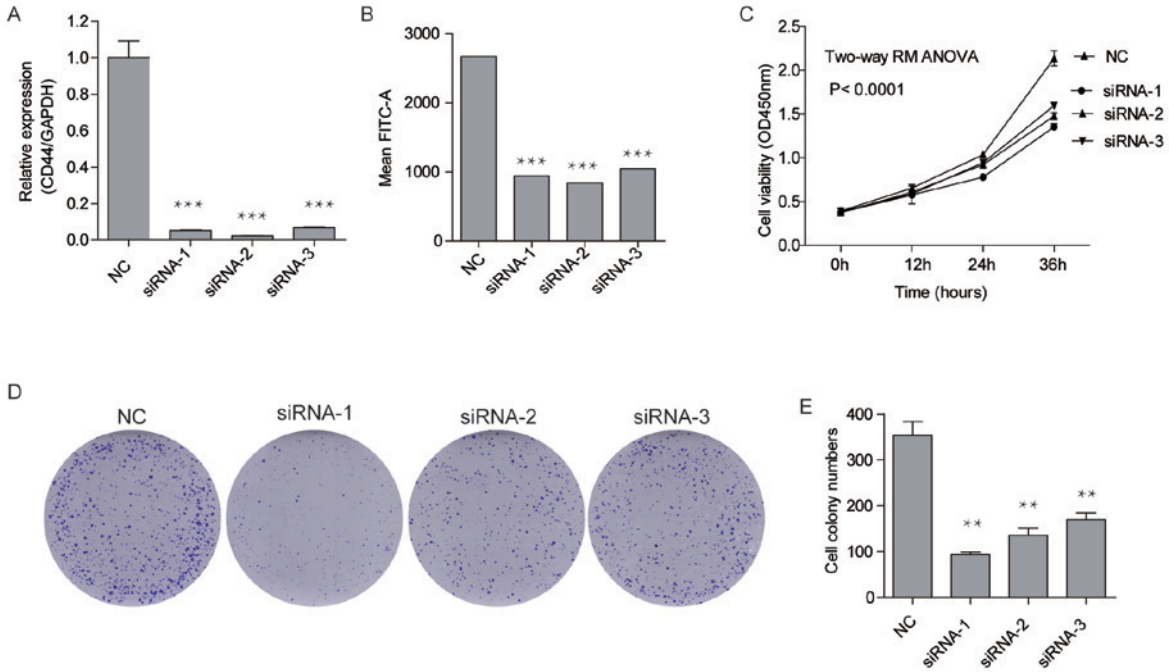

Figure 4. Effect of CD44 knockdown in A549 cells. (A) Reverse transcription-quantitative polymerase chain reaction and (B) flow cytometry analysis of CD44 expression in A549 cells transfected by siRNAs. (C) Cell Counting Kit-8 analysis of A549 cell proliferation following transfection with siRNAs. (D) Plate colony formation assay analysis of A549 cell proliferation following transfection with siRNAs. (E) Quantification of A549 cell colonies following transfection with siRNAs. Bars and error bars indicate the mean and standard deviation, respectively, from three different experiments. **P<0.01 and ***P<0.001. CD44, cluster of differentiation 44; siRNA, small interfering RNA; NC, negative control.

significant 12 h after seeding the cells. As time increased, the difference became increasingly significant, and the OD_{450} value of the H1299-CD44 cells was almost 2 times that of the H1299-control cells. These results confirmed our hypothesis that CD44 promoted cell proliferation *in vitro*.

Overexpression of CD44 improves the colony forming ability of H1299 cells. In order to further validate the hypothesis that CD44 promotes cell proliferation, a clonogenic assay was performed. A total of 500 cells were seeded on 60-mm plates and cultured for 8 days, with a medium changed each

3 days. As presented in Figs. 3D and E, the number of colonies of H1299-CD44 cells was significantly increased compared with H1299-control cells. In addition, the colonies formed by the H1299-CD44 cells were larger than those formed by the H1299-control cells (Fig. 3F). These results suggested that overexpression of CD44 improved the colony forming ability of H1299 cells.

Inhibition of CD44 suppresses the proliferation and colony formation ability of A549 cells. To further confirm this result, CD44 expression was knocked down by siRNAs in A549 cells with relatively high levels of CD44 expression (Fig. 2A). As presented in Fig. 4A, CD44 expression was decreased by 94.7, 97.7 and 93.2%, respectively for siRNA-1, siRNA-2 and siRNA-3, compared with cells transfected with siRNA-NC. Although the positive CD44 population of the A549 cells did not alter following siRNA transfection (data not shown), the average fluorescein intensity of CD44 also decreased by 64.6, 68.5 and 60.7% respectively for siRNA-1, siRNA-2 and siRNA-3, compared with cells transfected with siRNA-NC (Fig. 4B), indicating that CD44 expression was significantly inhibited by the siRNAs. As presented in Fig. 4C, A549 cells transfected with CD44 siRNA-1, siRNA-2 and siRNA-3 proliferated more slowly than the negative control cells ($P<0.05$). A clonogenic assay was then performed. The number of cell colonies formed by the siRNA-transfected cells was significantly decreased compared with the negative control cells (Fig. 4D and E; $P<0.01$). These results suggested that knockdown of CD44 expression suppressed A549 cell proliferation *in vitro*.

Discussion

In the present study, CD44 expression in NSCLC tissues was analyzed by immunohistochemistry, and a positive correlation between CD44 expression and T stage was identified ($P<0.05$). A stable CD44-overexpressing cell line, H1299-CD44 cells, and H1299-control cells, were then established. Using functional experiments, including CCK-8 assays and clonogenic assays, the data indicated that CD44 promoted NSCLC cell proliferation.

In previous studies, high expression of CD44 was hypothesized to be associated with poor prognosis in NSCLC (14,23). High expression of CD44 was also reported to be associated with not only occurrence and migration (15) but also metastasis (24) and drug resistance (19) of NSCLC. In the present study, an association between a higher CD44-positive expression rate and higher T stage was observed in NSCLC, which was consistent with the report from Shinohara *et al* (14) where significant associations were observed between CD44 expression and clinicopathological factors including T stage, N stage, pathological stage and histological type following immunohistochemical analysis of a cohort consisting of 261 consecutive patients (12), but the present study failed to identify a significant association between CD44 expression and occurrence or metastasis. This may be due to the small cohort in the present study, so a future study with a large cohort is still required to confirm the association between CD44 expression and other clinicopathological prognosis factors. There are also certain reports indicating that CD44 variant 6 (CD44v6) is associated with disease progression in NSCLC (13,25,26). Luo *et al* (23) reported that high expression of CD44v6 was associated with histopathological type and lymph node metastasis by using meta-analysis for a total of 921 patients with NSCLC from ten studies. However, whether the expression of specific CD44 isoforms predicts prognosis more effectively than total expression of CD44 is still worthy of future study. The data from the present study support that CD44 may be a useful prognostic marker for patients with NSCLC.

The present study provides direct evidence suggesting that CD44 overexpression increases proliferation, and knockdown of CD44 reduces proliferation in NSCLC cell lines. A series of experiments were conducted and the conclusion that CD44 promoted cell proliferation and growth *in vitro* was drawn. These results were constant with those of a previous report, where purified CD44-positive cells were demonstrated to have higher tumorigenicity than negative cells *in vitro* and *in vivo* (17). With increasing research focusing on the function of CD44 in cancer, it is generally accepted that CD44 is a prime therapeutic target for cancer interventions (27). The present study supports the hypothesis that CD44 is a potential therapeutic target for NSCLC. Inhibition of CD44 expression, either by siRNAs or by CRISPR/Cas9 gene editing targeting NSCLC, may suppress lung cancer cell proliferation and growth.

Collectively, the results of the present study suggest that CD44 overexpression is associated with an advanced T stage, and that CD44 may be a potential candidate target for the treatment of NSCLC, whereby CD44-dependent cell growth may be blocked. These results enhance our understanding of the involvement of CD44 in lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

BH performed the majority of the experiments. YM, YY, and LZH participated in certain experiments. HH and JC designed the experiments and coordinated the project.

Ethics approval and consent to participate

Written informed consent was obtained from all patients included in the present study, and this study was approved by the Ethics Committee of Peking University Cancer Hospital and Institute.

Consent for publication

All authors consented to publication.

Competing interests

The authors declare that they have no competing interests.

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