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THE STUDY OF THE TUMOR STEM CELL PROPERTIES OF $CD133^+CD44^+$ CELLS IN THE HUMAN LUNG ADENOCARCINOMA CELL LINE A549

H.Z. ZHANG[1], X.G. LIN[2], P. HUA[1], M. WANG[1], X. AO[1,*], L.H. XIONG[1], C. WU[3] AND J.J. GUO[1]

[1]Cardio-Thoracic surgery of the Sun Yat-sen Memorial Hospital, Sun Yat-sen university, Guangzhou 510120, China
[2]Department of oncology of the Sun Yat-sen Memorial Hospital, Sun Yat-sen university, Guangzhou 510120, China;
[3]Cardio-Thoracic surgery of the People's hospital of Ganzhou, Ganzhou 341000, China
Fax: +86-13632471129, Email: aoxiang78@163.com

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Abstract – We studied the tumor stem cell properties of the $CD133^+CD44^+$ subpopulation in the human lung adenocarcinoma cell line A549. A549 cells were classified into subpopulations based on differential expression patterns for CD133 and CD44. Cells from different subpopulations were cultured and subcutaneously injected into 32 nude mice. Our Results as following, (1) The majority of A549 cells died, whereas only about 4.11% of cells divided and proliferated to form cell clones. (2) The expression of CD133 and CD44 in proliferative cancer cells was statistically significantly different from that in normal A549 cells ($p < 0.001$). (3) Cell proliferation in group A ($CD133^+CD44^+$) was the fastest among all groups. Cell proliferation in A549 cells was slower than in group A but faster than in groups B ($CD133^-CD44^-$), C ($CD133^-CD44^+$), and D ($CD133^+CD44^-$). (4) The tumorigenic capacity in cells from group A was significantly higher than that in cells from groups B ($p<0.001$), C ($p<0.001$) and D ($p<0.04$). In conclusion, $CD133^+CD44^+$ cells in the adenocarcinoma cell line A549 have expressive significant cancer stem cell properties with continuous proliferative capacity and differentiation potential.

Key words: lung cancer, CD133, CD44, properties of tumor stem cells.

INTRODUCTION

In recent years, the incidence and mortality of lung cancer have increased significantly. This increase is mainly caused by difficulty in early diagnosis, early metastasis, and re-occurrence after treatment. Many studies found that the occurrence and development of malignant tumors are related to the presence of cancer stem cells. According to cancer stem cell theory, there is a rare population of cancer cells with stem cell properties in tumor tissue that maintain self-renewal and differentiation potential ability. This small group of cells is the resource and root for different differentiated cancer cells as well as tumor development. Recent studies on cancer-specific markers, such as CD133, CD44, and their variants, found that they are closely related to prognosis, early diagnosis, and invasive ability of tumors (2,7). CD133, a 120-kDa glycoprotein, is highly expressed in pluripotent adult stem cells. The expression of CD133 is closely related to lymph metastasis and prognosis in non-small cell lung cancer patients (12,14). CD44, a trans-membrane glycoprotein, is widely expressed on the cell surface and is an important adhesive protein related to malignant tumor metastasis. Its expression is related to cancer metastasis and invasion (4). There are two types of CD44 in human colorectal cancer specimens: standard (CD44s) and variant (CD44v). It was found that CD44v showed positive expression in invasive and metastatic colorectal cancer. In fact, CD44v is regarded as a cancer marker for colon cancer invasion (6). The human lung adenocarcinoma cell line A549 is a typical cell line model in lung adenocarcinoma studies. It has been widely used in lung cancer research for years. In this study, we used the A549 cell line to 1) screen for the continuously proliferative cancer cell subpopulation using a single-cell clone assay and 2) determine the expression patterns of CD133 and CD44 in continuously proliferative cancer cells and normal A549 cancer cells using an immunofluorescence assay and flow cytometry. Cells in each subgroup were amplified using a single-cell clone before subcutaneous injection into nude mice at different cell densities. The

cancer-forming abilities of these injected cells were observed and checked. In this study, we studied the cancer stem cell properties of the $CD133^+$ and $CD44^+$ subpopulations in the human adenocarcinoma A549 cell line.

MATERIALS AND METHODS

Materials

The rabbit anti-human CD133 monoclonal antibody, rabbit anti-human CD44 monoclonal antibody, goat anti-rabbit IgG-TRITC kit, immunohistochemical SABC kit, and MTT were purchased from Wuhan Bosde Biotech Company. The anti-CD44-FITC antibody was purchased from Ancell Company. The anti-CD133-PE antibody was bought from Miltenyi Biotec GmbH Company. The human adenocarcinoma A549 cell line and nude mice (BALA/c, 4-6 weeks old, 10-20 gram weight) were bought from the animal center of the School of Medicine, Sun Yat-sen University.

Methods

Part 1

A549 cells in liquid nitrogen were thawed immediately in a 37°C water bath. After thawing, A549 cells were centrifuged at low speed. The supernatant was poured off. Cells were rinsed in a PBS buffer one time. Then A549 cells were cultured in RPMI 160 media with 10% fetal calf serum (FCS) in a 37°C incubator containing 5% CO_2. When cells grew to the exponential phase, they were treated with trypsin, resuspended in culture medium, and adjusted to a density of $1x10^2$ cells/ml. The cells were evenly divided into a 96-well plate, with 1 cell in each well. Wells containing exactly 1 cell counted as valid wells. Six 96-well plates were inoculated, for a total of 511 valid wells. Cells were observed under an inverted microscope to examine cell growth, proliferation, and division. One week later, cancer cells with proliferative ability were serially transferred to 24-well plates, 6-well plates, and flasks for cell expansion. The cells were treated with trypsin to adjust the cell concentration to $1x10^6$ cells/ml. The cells were transferred by a Pasteur pipette, evenly spread onto glass slides within a 1-cm diameter, and incubated at 37°C for 60 minutes. Then the cells were incubated with the first antibody (rabbit anti-human monoclonal antibody, diluted 1:200 in PBS buffer) at 4 °C overnight. After three washes with PBS buffer, the cells were incubated with the second antibody (goat anti-rabbit IgG-TRITC kit, diluted 1:400 in PBS buffer) at 37°C for 30 minutes. The negative control group was incubated with PBS buffer instead of the first antibody. Normal A549 cancer cells were used as an internal control (see the manual for more details). The cells were then sorted into four subgroups: $CD133^+CD44^+$ (group A), $CD133^-CD44^-$ (group B), $CD133^-CD44^+$ (group C), and $CD133^+CD44^-$ (group D). The sorted cells were cultured in RPMI 1640 with 10% FCS without a differentiation inhibitor reagent in a 37 °C incubator containing 5% CO_2 overnight. Healthy, growing cells in each group and the normal A549 group were treated with trypsin to make a single cell resuspension and permit counting of cell numbers. A total of 20 ul of MTT (5 mg/ml) was added into 10 wells in each group. Cells were incubated at 37 °C for 4 hours before the supernatant was removed. A total of 150 μl of DMSO was added to each well, and the wells were then shaken for 10 minutes. The OD value of each well was read at 490 nm using an enzyme immunoassay method. The above steps were repeated for cultures on days 2, 3, 4, 5, 6, and 7. Cell growth was monitored based on the OD value on different culture days. Flow cytometry was used to examine the self-renewal and differentiation ability of cells in the four subgroups and to monitor the cell phenotype change after proliferation in each subgroup. A total of $1x10^2$ and $1x10^5$ healthy cells from each subgroup were injected into the skin of each nude mouse at two injection sites. A total of 32 mice with 64 injection sites were used. Tumorigenesis and metastasis were examined after 4 to 6 weeks. Cell phenotypes were examined by staining the tumor tissue with CD133 and CD44 antibodies in an immunohistochemical study (SABC method).

Part 2

RNAi experiment for CD133+ and CD44+ cells

1: Three siRNAs were selected for CD44 and CD133 respectively.

[Product name:si CD44-h_001]

Product Code:siB0977132156
Target sequence: CCAGCAAGTCTCAGGAAAT

[Product name:si CD44-h_002]
Product Code:siB0977132243
Target sequence:GCAGTCAACAGTCGAAGAA

[Product name:si CD44-h_003]
Product Code:siB07122116562
Target sequence:TTCCAGAATGGCTGATCAT

[Product name:si CD133-h_001]
Product Code:siG09218120132
Target sequence:GCCAGAAACTGTAATCTTA

[Product name:si CD133-h_002]
Product Code:siG09218120154
Target sequence:GGAGAACAATAATAGGATA

[Product name:si CD133-h_003]
Product Code:siG09218120214
Target sequence:GTGAACATCTCAACATTAA

2: A549 cells were seeded into six 6-well plates with a density of 5 x 10^4 cells/well.

Sample Settings:

Plate 1:

Negative_Cy3 siRNA_25nM	Negative_Cy3 siRNA_50nM	Negative_Cy3 siRNA 75nM
Negative NC siRNA_25nM	Negative NC siRNA_50nM	Negative NC siRNA_75nM

Plate 2:

siCD44-h siRNA_25nM	siCD44-h siRNA_50nM	siCD44-h siRNA_75nM
siCD133-h siRNA_25nM	siCD133-h siRNA_50nM	siCD133-h siRNA_75nM

Interference efficiency testing

siRNA interference efficiency testing at RNA levels

The transfection was conducted at the second day when cells reached 70% confluence. After 48 h transfection, the culture medium was withdrawn and 1 ml Trizol was added into each well for total RNA extraction. To test the RNA intactness, 1 μl RNA extraction was diluted by 60 times and examined on a 520UV/Vis Spectrophotometer. The ratio of OD260/OD280 was higher than 1.8 and demonstrated an intact RNA extraction without protein pollution. To test the RNA integrity, 1 μl from each RNA extractant, was electrophoresed on 1% agarose gel at 80 V for 20 min. A gel imaging system (Landbiology, China, Guangzhou) was used to observe the 5s rRNA, 18s rRNA, and 28s rRNA fragments of the total RNA.

Stem-loop reverse transcription PCR was performed with the following conditions:

The sequences included the internal reference of 18S rRNA, 112 bp and the target sequence of CD44, 293 bp.

The primers used are qh-CD44-F1, 5'-tcaagcctggtagaattggc-3' and qh-CD44-R1, 5'-acagtggtcattgtacagca-3'.

The reaction system consisted of cDNA (1:15) 5.0 μl, up-stream primer 0.5 μl, down-stream primer, 0.5 μl, 2 × SYBR green PCR master mix 10 μl, and H_2O 4.0 μl. The reaction was performed as follows, 95^{o}C 5min, and then 40 cycles including 95^{o}C 15s, 60^{o}C 15s, and 72^{o}C 20s. The melting curve was analyzed at 60^{o}C-95^{o}C once per 0.4^{o}C. Each sample was analyzed for three times.

siRNA interference efficiency testing at protein levels by Western Blotting

The siRNA transfection group with the highest efficiency was chosen for western blotting according to the above RT-PCR. The samples collected above were electrophoresed in 10% SDS-PAGE and then electro-transferred onto polyvinylidene fluoride membrane. Primary antibody (1:1000) was incubated at 4°C overnight. After incubation of secondary antibody at 37°C for 1 h, chemiluminescent substrate was added for 5 min. After exposure, the film was scanned.

Proliferation ability after the knock-down of CD44 expression

The MTS [3-(4,5-dimenthylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium] method and cell counting method was used to detect the cell of each group

Statistical analysis

Under a fluorescence microscope, cells with red particles in their membrane or cytoplasm were denoted as positive. A total of 200 cancer cells were counted within ten high-magnification microscope visual fields. The final results were made by two pathologists after independent observation of the slides. Fisher's Exact Test was used to compare the tumorigenic ratio between groups. P-values were considered to be significant at values less than 0.05. The data were processed and analyzed using SPSS11.5 software.

RESULTS

Proliferation and division ability of the human adenocarcinoma cell line A549

After one week of culture, only 4.11% of A549 cells (21 out of 511 wells) showed continuous self-renewal ability (Fig. 1). Fig. 2 shows the curvatures for cell division and death in single A549 cells on different culture days.

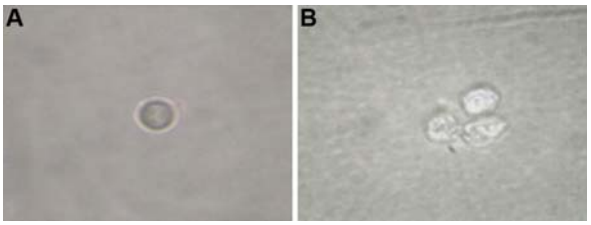

Figure 1. (A) The hole contain one cell is valid (×200). (B). Cell division after 48 hours (×200)

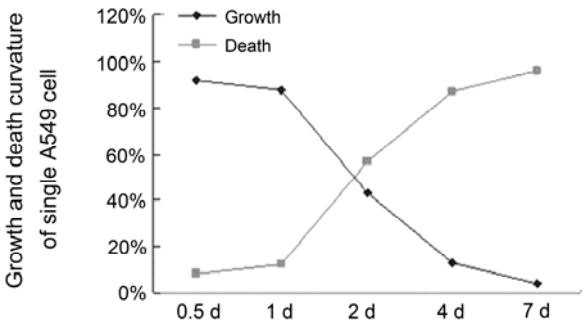

Figure 2. Growth and death curvature of single A549 cell

Cell immunofluorescence staining results

The results of immunofluorescence staining on cells with continuous proliferative capability were as follows. Roughly 19% of cells were CD133-positive (Fig.3), and 57% were CD44-positive (Fig.3).

The results of immunofluorescence staining on normal A549 cells were as follows. Roughly 2.5% of cells were CD133-positive (Fig.3), and 34% were CD44-positive.

A χ^2 test revealed that the proportions of cells positive for CD133 and CD44 in continuously proliferating tumor cells were significantly higher than those in the normal A549 cells (p-value less than 0.001, Table 1).

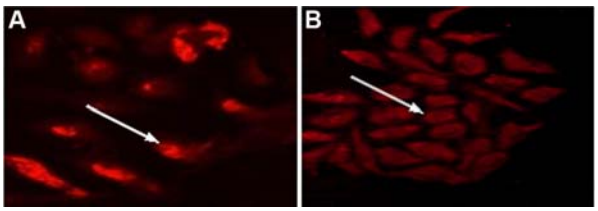

Figure 3. (A) CD133$^+$ cells with red staining around membranous and cytoplasm (×200). (B) CD44$^+$ cells with red staining around membranous and cytoplasm (×200).

Cell proliferative ability analysis in each group

Four different cell subgroups, including CD133$^+$CD44$^+$ (group A), CD133$^-$CD44$^-$ (group

B), $CD133^{-}CD44^{+}$ (group C), and $CD133^{+}CD44^{-}$ (group D), were sorted using flow cytometry (Fig.4). Cells in each group were amplified and analyzed using flow cytometry to determine downstream cell phenotypes. Cells in groups B, C, and D demonstrated no differentiation capacities, whereas cells in group A could differentiate into downstream subpopulations. Cell growth was detected using the MTT method. Cells from all groups demonstrated proliferative abilities. Cells from group A grow the fastest. Cells from the normal A549 group grow slower than those in group A but faster than those in groups B, C, and D (Fig.5).

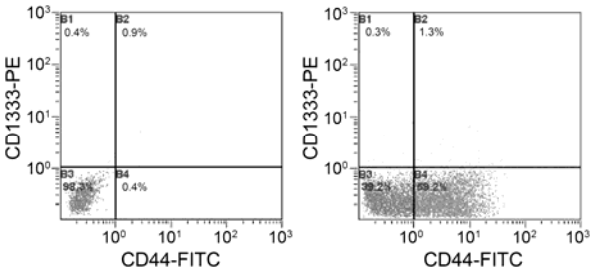

Figure 4. (A) Flow cytometry of A549 cells. (B) Flow cytometry of $CD133^{+}CD44^{+}$ cells.

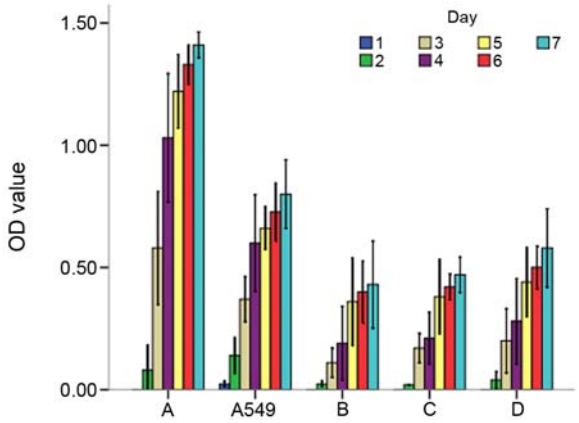

Figure 5. Growth curvature of each group.

Analysis of cell tumorigenesis ability for each group in nude mice

According to the Fisher's Exact Test, the tumorigenic ability of group A cells at densities of $1x10^{2}$ and $1x10^{5}$ was significantly higher than that in cells from groups B, C, and D ($p<0.001$, $p<0.001$, and $p<0.04$, respectively, Table 2). Tumor tissue was confirmed to be adenocarcinoma by pathological diagnosis, and no metastasis was found in other organs. Pathological immunohistochemical analysis with anti-CD133 and anti-CD44 antibodies revealed that transplant tumors contained cells with all phenotypes except that of group A cells.

Table 1. Expression of CD133 and CD44 proteins in different cell groups

Group	CD133		CD44	
	+	-	+	-
Cells that can proliferate and divide continuously	38	162	114	86
Normal cells	5	195	68	132

Table 2. Tumorigenesis ability of cells from each group

Group	Cell density	
	1×10^{2}	1×10^{5}
A	3/8	6/8
B	0/8	0/8
C	0/8	1/8
D	0/8	1/8

Interference efficiency testing results

1. Testing of the transfection efficiency (cyc3 observation, Fig. 6). Gel images for each interference group contained 5s rRNA, 18s rRNA, and 28s rRNA. This demonstrated the total RNA extraction was intact (Fig. 7, 8).

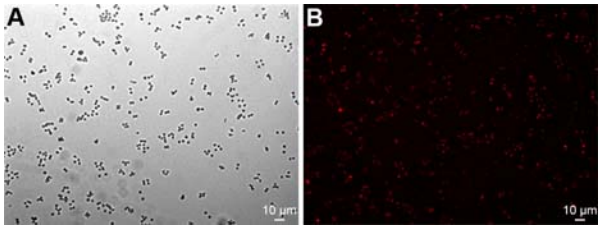

Figure 6. (A) A549 cells transfected with 50 nM siRNA in a bright field. (B) A549 cells transfected with 50 nM siRNA in a dark field (the bright red color indicates successfully transfected cells). The transfection efficiency met the requirements of this study.

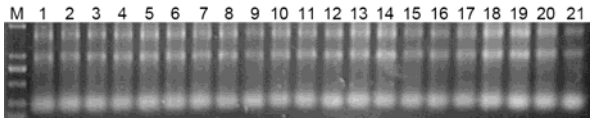

Figure 7. Lane1:CD44-1 25; Lane2:CD44-1 50; Lane3:CD44-1 75; Lane4:CD44-2 25; Lane5:CD44-2 50; Lane6:CD44-2 75; Lane7:CD44-3 25; Lane8:CD44-3 50; Lane9:CD44-3 75; Lane10:NC 25; Lane11:NC 50; Lane12:NC 75; Lane13:CD133-1 25; Lane14:CD133-1 50; Lane15:CD133-1 75; Lane16:CD133-2 25; Lane17:CD133-2 50; Lane18:CD133-2 75; Lane19:CD133-3 25; Lane20:CD133-3 50; Lane21:CD133-3 75; M: Marker (100, 250, 500, 750, 1,000, and 2,000 bp from bottom to up).

2. The histogram of RNA ct values showed that the optimum sequences and concentrations for siRNA transfection efficiency were si CD44-h_001 25nM and si CD133-h_001 25nM (Fig. 9). Stably transfected cells were obtained through screening.

3: siRNA interference efficiency tested by western blotting. It was shown that the CD133 and CD44 expression was significantly suppressed in A549 cells transfected with siRNAs (normalized against non-transfection expression, $P < 0.01$). And this met the experimental design requirements.(Fig. 10-12)

Cell proliferations after the CD133 and CD44 expression was suppressed

Cultured human lung cancer cell lines A549 were used for the test. The non-transfection groups included CD133+CD44+ (group A), CD133-CD44- (group B), and unsorted A549 cells (group C). The transfect groups included CD44-CD133- (group D) and negative controls (NC, group E). (Fig. 13)

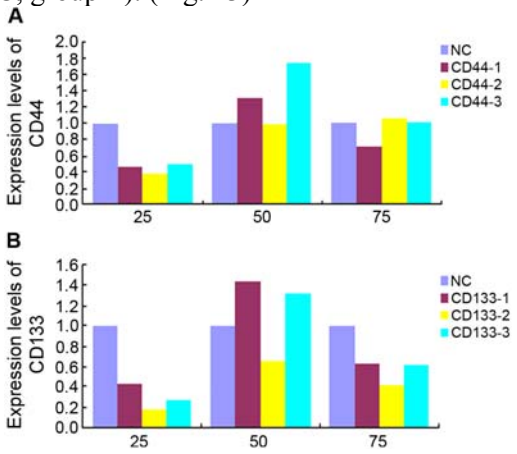

Figure 9. (A) Expression levels of CD44 in each sample of the three groups. (B) Expression levels of CD133 in each sample of the three groups. Melting curves showed the single-peaks, indicating a high specificity and accurate quantification. The histogram of RNA ct values showed that the optimum fragment and concentration for siRNA transfection efficiency is 25 nM. Stably transfected cells were obtained through screening.

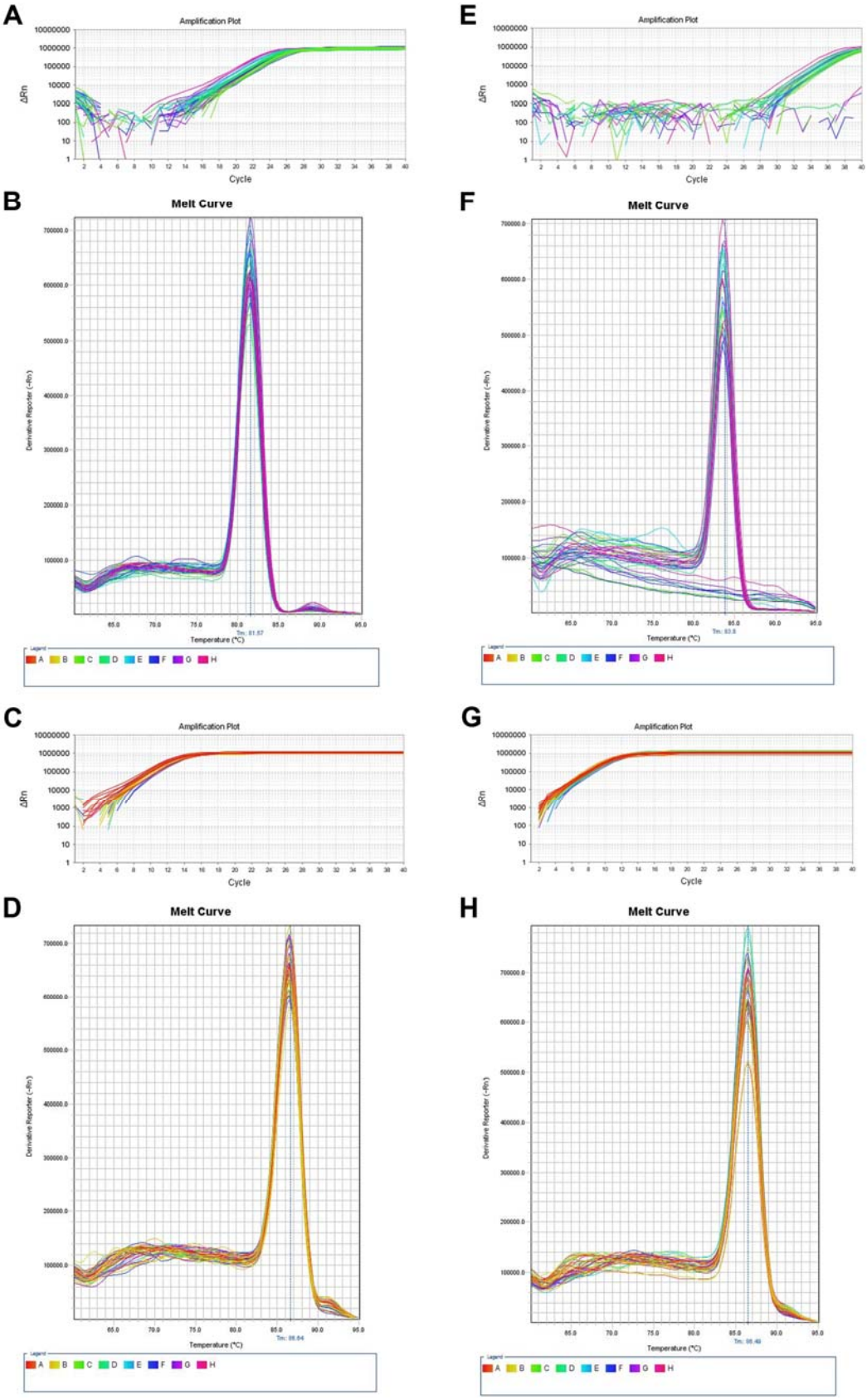

Figure 8. (A) The CD44 amplification curve. (B) The CD44 melting curve. (C) The 18s amplification curve of controls. (D) The 18s melting curve of controls. (E) The CD133 amplification curve. (F) The CD133 melting curve. (H) The 18s amplification curve of controls. 8(I) The 18s melting curve of controls. The electrophoresis of RNA extract showed complete 5s rRNA, 18s rRNA, and 28s rRNA bands, which demonstrated an intact RNA extraction. The single peak melting curve showed a good specificity and accurate quantification.

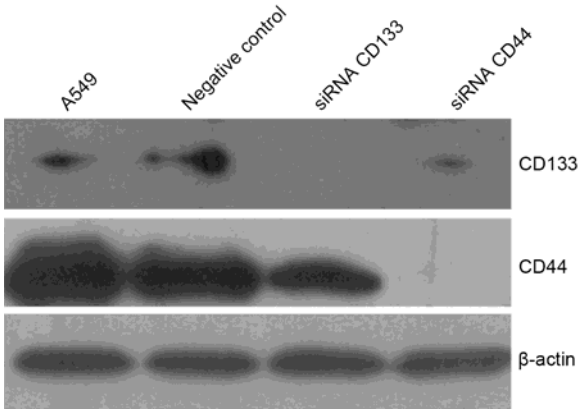

Figure 10. 1:A549 cells 2:NC negative control 3:siRNA CD133 4:siRNA CD44
The efficiency of siRNA interference was determined by western blotting. After transfection, the expression of CD44 and CD133 was significantly down-regulated ($P < 0.01$). There was significant difference in the ratio of gray values before and after the transfection between the two groups ($P < 0.01$). This met the requirements of this study.

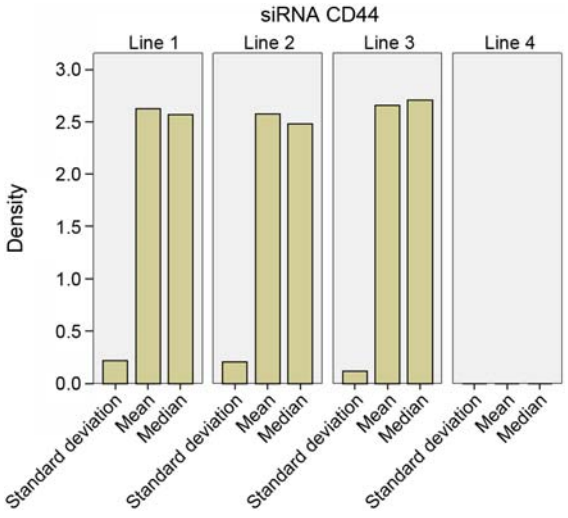

Figure 11. The relative gray values of siRNA CD44 showed there was no significant difference among line 1, 2 and 3 ($p>0.05$). However, line 4 had a significant reduction compared with the other 3 lines (all $p<0.01$).

DISCUSSION

Tumors are formed due to the effects of multiple tumorigenic factors once cells lose their normal control of cell division and proliferate indefinitely. The formation and metastasis of malignant tumors are complex processes that are regulated and controlled by many factors. These include the expression and regulation of metastasis genes and metastasis inhibitor genes, the malignant proliferation of cancer cells, angiogenesis during tumor development, the expression of adhesion protein by cancer cells, cancer cell migration, and cancer cell avoidance of the body's immune system. How to control the metastasis of tumor cells is a hot field in cancer research. Recent studies suggest that there is a small population of cells with stem cell properties that is important during cancer formation and development. These cells demonstrate self-renewal and multipotency, and they are believed to be the source of different differentiated cancer cells as well as the root of continuous tumor growth. This small cell population is referred to as the tumor stem cell (TSC) population. During tumorigenesis, the expression of different genes causes different differentiated cancer cells to express different molecular markers on their cell surfaces. We hoped to screen these TSCs according their specific cell surface antigens. Based on previous studies (13,15) by ourselves and others, we find that the expression of CD133 and CD44 is significantly higher in non-small lung cancer than other normal tissues. CD133 and CD44 are primarily regarded as lung cancer cell surface markers. The expression of CD133 and CD44 on continuously proliferating cell surfaces is studied in the current work. The human lung adenocarcinoma cell line A549 is a traditional lung cancer model that has been widely used in lung cancer research for years. In this study, the A549 cell line was used for single cell cloning, screening of continuously proliferating cancer cells, and detecting the expression of CD133 and CD44 using flow cytometry and immunofluorescence staining. Further, we studied the TSC properties of $CD133^+$ $CD44^+$ cells. This study will promote markers identifying lung tumor stem cells.

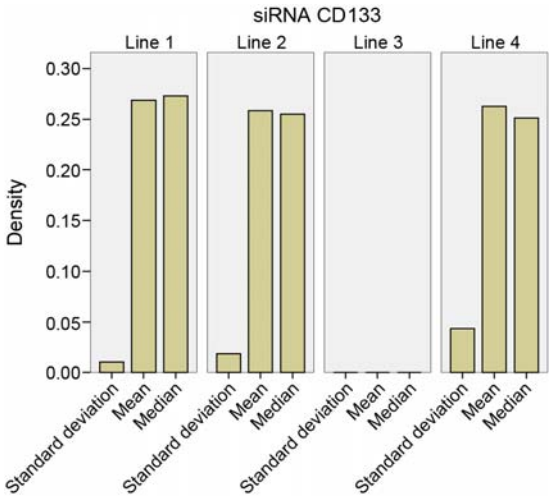

Figure 12. The relative grave values of siRNA CD133 showed that there was no significant difference among line 1, 2 and 4 ($p>0.05$). However, line 3 had a significant reduction compared with the other 3 lines (all $p<0.01$).

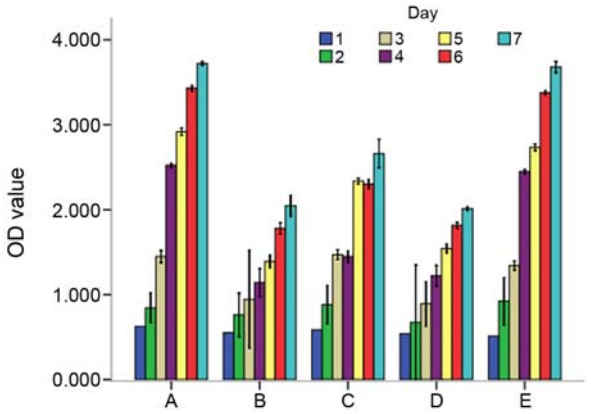

Figure 13. The MTS method and cell counting method was used to detect the cell proliferation ability of each group after the A549 cells were successfully transfected with siRNAs. The MTS curve showed that the cell viability significantly decreased after transfection. The line of the transfected group drew close to the line of the non-transfection negative expression group. The growth curve of the transfected group obviously tended to be flat.

CD133 is a 120 kDa glycoprotein with five transmembrane domains. CD133 was first found in hematopoietic stem cells and hematopoietic progenitor cells. It is confirmed that CD133 is also a marker of stem cells in the non-hematopoietic system. It is expressed in progenitor cells in endothelial and nerve tissues but is not expressed in mature blood cells, endothelial cells, or nerve cells. It is also expressed in multipotent adult stem cells (9). CD133 is a specific antigen expressed on stem cell surfaces that is lacking on mature cells. Singh (11) found that some cells in human brain tumor tissue expressed the neuron stem cell marker CD133. Purified $CD133^+$ tumor cells show strong self-renewal and differentiation abilities. These cells can also reconstruct the phenotypes of other cells from the original tumor. They contain properties belonging to stem cells. Therefore, CD133 is regarded as a specific marker for brain tumor-originating cells. Recent studies demonstrate that CD133 is also a stem cell surface marker in human prostate cancer (3) and colon cancer (8). We also found that the expression of CD133 was closely related to the migration of lymph cells and prognosis in non-small lung cancer patients (13).

CD44 is an important adhesion molecule related to malignant tumor metastasis (6). The CD44 gene localizes to the small arm of human chromosome 11. It contains at least 20 exons, 10 of which constantly are expressed mature mRNAs. Other exons are inserted into pro-mRNAs through selective splicing to encode different CD44 proteins. There are two kinds of CD44 protein: standard (CD44s) and variant (CD44v). In normal function, CD44 is the receptor for hyaluronic acid (HA) and collagen (5,10). CD44 mainly correlates with lymph cell activation, specific adhesion between cells, and specific adhesion between cells and the extracellular matrix. Mutations in and variant expression of CD44 are closely related to cancer growth and metastasis (6). A study in 136 breast cancer specimens by Ibraham (1) demonstrated that the number of $CD44^+/CD24^{-/LOW}$ cancer cells had no relationship with the final survival rate of cancer patients but did show a relationship with tumor metastasis as well as special metastasis in bone. Higher $CD44^+/CD24^{-/LOW}$ cell numbers were associated with higher rates of metastasis. This study confirms the idea that expression of CD44 is very closely related to tumor metastasis and invasion. This research confirms the fact that CD44 and its variants have great potential value in tumor prediction, early diagnosis, tumor metastatic potency, and prognosis. The detection of CD44 protein is therefore becoming increasingly important in cancer research.

Based on tumor stem cell theory, TSCs are a kind of cell with an indefinite passaging ability. They can continuously proliferate and divide. TSCs are have been identified as key factors for tumor growth, metastasis, and invasion. Subcutaneous inoculation of cancer cells is a common method in animal transplant tumor models. There are plenty of blood vessels under the oxter of nude mice forelimbs, which generates good conditions for the survival of transplant tumors observation of tumor growth. Therefore, cells screened from each subpopulation are inoculated under the oxter of nude mice forelimbs to permit observation of the tumorigenesis ability of each subpopulation. We used the A549 cell line for single-cell cloning, screening of continuously proliferating cancer cells, and detecting the expression of CD133 and CD44 using flow cytometry and immunofluorescence staining. We found that only 4.11% of A549 cells could continuously proliferate and divide. The majority of A549 cells died after being cultured for a while. This finding indicates that the majority of A549 cells are mature tumor cells that cannot survive for a long time and cannot differentiate. Only a small population of A549 cells can survive for a long time, continuously proliferate, and divide. This means that the passaging of the A549 cell line is only related to this small population of cells. The

majority of A549 cells lose their ability to continuously proliferate and divide. Through immunofluorescence technology, we found that a very small fraction of A549 cells is $CD133^+CD44^+$. Cell growth was detected using the MTT method. Fig.5 demonstrates that cells from all groups have proliferative abilities. Cells from group A grow the fastest. Cells from the normal A549 group grow slower than those in group A but faster than those in groups B, C, and D. This finding indicates that if A549 cells were depleted of group A cells, proliferative ability would decrease dramatically in the remaining cells. During the amplification and passaging of each group of cells, we noted that cells from groups B, C, and D could not differentiate into other cell subtypes; in contrast, cells from group A could differentiate into other cell subtypes. We compared the proliferation ability of A549 cells before and after the expression of CD133 and CD44 were down-regulated by RNAi. The MTS results showed the proliferation ability of the A549 cells was significantly pressed and that the cell proliferation and grow curves of the transfected groups nearly merged with that of the non-transfected CD133-CD44- group. In the tumor tissue from nude mice inoculated with group A cells, all cell phenotypes with the exception of the group A cell phenotype were found. This finding further indicates that $CD133^+CD44^+$ cells have self-renewal and differentiation capabilities. In this paper, the study of biological characteristics of $CD133^+CD44^+$ cells in the human lung adenocarcinoma cell line A549 provides the basis for determining whether there are tumor stem cells in lung cancer tissue. Additionally, we provide evidence for the identification of lung cancer stem cell surface markers and further suggest new ideas and directions for clarifying the mechanisms of lung cancer formation and successfully treating lung cancer in the clinic.

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